HIGH THROUGHPUT DIRECTED EVOLUTION BY RATIONAL MUTAGENESIS

RELATED APPLICATIONS

Benefit of priority under 35 U.S.C. §119(e) is claimed to U.S.

5 provisional application Serial No. 60/315,382, filed August 27, 2001, to Manuel Vega, Lila Drittanti and Marjorie Flaux, entitled "HIGH THROUGH-PUT DIRECTED EVOLUTION BY RATIONAL MUTAGENESIS." The subject matter of this application is incorporated in its entirety by reference thereto.

FIELD OF INVENTION

10 Processes and systems for the high throughput directed evolution of peptides and proteins, particularly those that act in complex biological settings, are provided. The proteins and peptides include, but are not limited to, intracellular proteins, messenger/signaling/hormone proteins and viral proteins.

15 BACKGROUND

Directed evolution refers to biotechnological processes for optimizing the activity of proteins by means of random changes introduced into selected respective genes. Directed evolution involves the creation of a library of mutated genes, and then selection of the mutants

20 that encode proteins having desired properties. The process can be an iterative one in which gene products that have improvement in a desired property are subjected to further cycles of mutation and screening. Directed evolution provides a way to adapt natural proteins to work in new chemical or biological environments, and/or to elicit new functions.

25 The potential plasticity of proteins is such that chances exist that for every new challenge, such as a new environment and desired new or altered activity, it should be possible, given a sufficient pool of modified proteins (or encoding nucleic acids), that an appropriately 'evolved' protein could be found that would have a desired activity. The problem is

30 in generating and then identifying the appropriate sequence.

There have been practical approaches to this problem (see, *e.g.*, U.S. Patent Nos. 6,096,548; 6,117,679; 6,165,793; 6,180,406;

6,132,970; 6,171,820; 6,238,884; 6,174,673; 6,057,103; 6,001,574; 5,763,239; 5,837,500; 5,571,698; 6,156,509; 5,723,323; 5,862,514; 5,871,974; 5,779,434 and others). Typically theses approaches are of two types. One is a purely "rational" approach that is based on the

5   assumption that the optimized proteins can be rationally designed. This, however, requires sufficient information regarding the laws that govern protein folding, molecular interactions, intra-molecular forces and other dynamics of protein activity. This rational approach is extremely dependent on a number of variables and parameters that are not known.

10   Consequently, although useful in some specific cases and applications, the rational approach intended to 'predict' protein structure remains limited in applicability.

In contrast to the rational approach, random approaches have also been employed. One random approach requires synthesis of all possible

15   protein sequences or a statistically sufficient large number of proteins and then screening them to identify proteins having the desired activity or property. Since the resources to synthesize all possible theoretical sequences of a single protein is not possible, this approach is impracticable. Other random approaches are based on gene shuffling

20   methods, which are PCR-based methods that generate random rearrangements between two or more sequence-related genes to randomly generate variants of the gene.

The development and scope of directed evolution, thus, has been limited, and its potential remains to be exploited. In order to exploit the

25   potential of directed evolution, alternative approaches for generating and identifying evolved proteins are needed. It is an object herein to provide methods and products to exploit the potential of directed evolution.

SUMMARY

Provided herein are methods for performing directed evolution for

30   the optimization of proteins that function in complex biological settings. Methods of high throughput directed evolution of proteins are provided.

In practicing the methods, each molecule is individually designed, produced, processed, screened and tested in a high throughput format. Neither random or combinatorial methods nor mixtures of molecules are used.

5   The methods provided herein include the steps of identifying a protein target of interest; obtaining nucleic acids that encode the target, which may be from any source, such as a natural library, a collection generated by known gene shuffling techniques and related methods, and, then creating variants of the proteins using methods for rational

10   mutagenesis provided herein.   Whatever method is used to select or generate the nucleic acids encoding the protein targets, each molecule is processed and screened separately in a high throughput format.

        The nucleic acids encoding each variant are individually screened. They can be screened in any suitable assay, including cell-based assays

15   and biochemical assays. For cell based assays, each nucleic acid molecule is introduced into an expression vector for expression in a bacterial cell or into a vector for expression in a eukaryotic host cell. In all instances, the nucleic acids of interest are introduced into host cells in an expression vector, such as by transfection for bacterial hosts and

20   transduction with viral vectors into eukaryotic hosts with viral vectors.

        Each variant is introduced into a host and the resulting cells are maintained separately, such as in an addressable array of wells in a microtiter plate or other substrate with discrete locations for performing reactions or retaining molecules of interest. Typical formats are 96 loci,

25   and multiples thereof (384, 1536, 3072, . . . 96 x n, where n is 1 to any number desired, such as 10, 20, 30, 50 . . .100), although any convenient number of loci may be employed.

        Since the process is conducted in a high throughput format, for many embodiments, it is often important to assess the relative numbers

30   of transformed, transduced or transfected cells. Hence the relative (or actual) titer of the vector, such as the recombinant viral vector, must be

known to permit analysis of results. For high throughput formats, it is important to assess the relative or actual concentration of the viral vector (or plasmid) so that results can be compared among all cells and variants. Methods for titering (determining the concentration) of the nucleic acid

5   encoding the variant and/or the recombinant virus are also provided.

The processes require accurate titering of the viruses in a collection or among collections (libraries) so that the activities of the screened mutant proteins can be compared. Provided are general methods for the quantitative assessment of the parameters of activity corresponding to

10 the individual variants in the library, based upon intracellular serial dilution generated by precise tittering with the gene transfer viral vectors Any method permits accurate titering may be used, including that described nInternational PCT application No. PCT/FR01/01366, based on French application n° 0005852, filed 9 May 2000, and published as International

15 PCT application No. WO 01/186291. A method of titering, designated Tagged Replication and Expression Enhancement Technology (TREE") is provided herein.

Each of the different cells is separately screened by a suitable assay, and the results analyzed. Methods for assessing the interactions

20 in biological systems, such as a Hill-based analysis (see, published International PCT application No. WO 01/44809 based on PCT n° PCT/FR00/03503, Dec, 2000, and the description herein), or a second order polynomial or other algorithm that describes the interaction between cells and biological agents to select variants that have a desired property

25 are employed in the processes herein.

A semi-rational method for evolution of proteins that is particularly designed for use in the methods herein or in any method that uses "evolved" proteins is also provided. The method, which is based on an amino-acid scanning protocol, is for rationally designing the variants for

30 use in the directed evolution and selection method, and can employ iterative processing of the steps of the high throughput methods provided

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herein. In this method, once the target protein or domain is identified, nucleic acid molecules encoding variants are prepared. Each variant encoded by the nucleic acid molecules has a single amino acid replaced with another selected amino acid, such as alanine (Ala), glycine (Gly),

5   serine (Ser) or any other suitable amino acid, typically one selected to have a neutral effect on secondary and tertiary structure. The resulting series of variants are separately screened in the high throughput format provided herein, and those that have a change in the target activity are selected and the modified amino acids are designated "hits." Nucleic acid

10   molecules encoding proteins in which each hit position is replaced by the eighteen remaining amino acids then are synthesized and the resulting collection of molecules are screened, such by introduction into host cells, and the proteins that result in a improvement of a targeted activity, are identified. Such proteins are designated "leads." Leads may be further

15   modified by producing proteins that have combinations of the mutations identified in the leads. This method, which does not require any knowledge of the structure of a target protein, permits precise control of locations where changes are introduced and also the amount of change that is introduced.

20   The high throughput directed evolution processes provided herein include the use of virus libraries containing mutant versions of a gene; viral libraries of such mutant genes are also provided.

Reporter cells are infected with the titered viruses that encode the mutant genes. The mutant genes are expressed and read-out data from

25   either biochemical or cell-based assays, while isolating each mutant/virus physically from the others (i.e. one-by-one analysis), are collected and analyzed. Serial dilution assays (i.e. a series of dilutions for each individual mutant/virus in the library) are used and the biochemical/cell-based assays are performed on each single dilution for each individual

30   mutant/virus. Analysis of the serial dilution readout-data can be performed using any method of analysis that permits one-by-one

comparisons. Hill-based analysis (see, published International PCT application No. WO 01/44809 based on PCT n° PCT/FR00/03503, Dec, 2000, and the description herein) are employed for analysis of the data.

Protein/protein domain variants identified using the methods are also provided. Also provided are nucleic acid molecules and proteins and polypeptides produced by the methods and viruses and cells that contain the nucleic acid moleucles and proteins.

In an exemplary embodiment of methods provided herein, the process of rational directed evolution provided herein is applied to the AAV rep gene. The resulting recombinant rep protein variants and rAAV are also provided. Among the rep proteins are those that result in increased rAAV production in rAAV that encode such mutants, thereby, among a variety of advantages, offering a solution to the need in the gene therapy industry to increase the production therapeutic vectors without up-scaling manufacturing.

Thus, for exemplification, some methods provided herein have been used to identify amino acid "hit" positions in adeno-associated virus (AAV) rep proteins that are relevant for AAV or rAAV production. Those amino acid positions are such that a change in the amino acid leads to a change in protein activity either to lower activity or to higher activity compared to native-sequence Rep proteins. The hit positions were then used to generate further mutants designated "leads." Provided herein are the resulting mutant rep proteins that result in either higher or lower levels of AAV or rAAV virus compared to the wild-type (native) Rep protein(s).

In addition to enhancing AAV production, among the rep mutants are those that inhibit papillomavirus (PV) and PV-associated diseases, including certain cancers and human immunodeficiency virus (HIV) and HIV-associated diseases.

Systems and computer controlled systems for performing the high throughput processes are also provided.

DESCRIPTION OF THE FIGURES

FIGURES 1 summarize various exemplary embodiments of the high throughput processes provided herein. FIGURE 1A depicts an embodiment of the process in which an amino acid scan is employed to

5   generate a library of mutants, which are then introduced into viral vectors, such as an adeno-associated viral vector (AAV), an herpes virus, such as herpes simplex virus (HSV) and other herpes virus vectors, a vaccinia virus vector, retroviral vectors, such as MuMLV, MoMLV, feline leukemia virus, and HIV and other lentiviruses, adenovirus vectors and

10 other suitable viral vector, each member of the library is individually tested and phenotypically characterized to identify HITS. FIGURE 1B summarizes round 2 in which LEADS are developed by mutagenesis at and/or surrounding the positions identified as HITS; FIGURE 1C summarizes the optional next round in which recombination among

15 LEADS is performed to further optimize the LEADS; FIGURE 1D depicts the process in mammalian cells; and FIGURE 1E depicts the process in bacterial cells.

FIGURE 2A depicts an exemplary titering process (in this instance the TREE" for titering AAV) in a 96 well format; FIGURE 2B shows the

20 results and analysis of a titering process performed using the TREE" procedure; and FIGURE 2C shows an exemplary calibration curve for the calculation of the titer using the TREE" method.

FIGURE 3A and 3B depict "HITS" and "LEADS" respectively for identification of AAV rep mutants "evolved" for increased activity.

25 FIGURE 4 shows the genetic map of AAV, including the location of promoters, and transcripts; amino acid 1 of the Rep 78 gene is at nucleotide 321 in the AAV-2 genome.

FIGURES 5A and 5B show the alignment of amino acid sequences of Rep78 among AAV-1; AAV-6; AAV-3; AAV-3B; AAV-4; AAV-2; AAV-

30 5 sequences, respectively; the hit positions with 100 percent homology among the serotypes are bolded italics, where the position is different

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(compared to AAV-2, no. 6 in the Figure) in a particular serotype, it is in bold; a sequence indicating relative conservation of sequences among the serotypes is labeled "C".

Legend:

5   1 is AAV-1; 2 is AAV-6, 3 is AAV-3, 4 is AAV-3B,
               5 is AAV-4, 6 is AAV-2, and 7 is AAV-5;
               "." where the amino acid is present ≥ 20%;
               ":" where the amino acid is present ≥ 40%;
               "+" where the amino acid is present ≥ 60%;
10   "*" where the amino acid is present ≥ 80%; and
               where the amino acid is the same amongst all
               serotypes depicted it is represented by its single letter
               code.

DETAILED DESCRIPTION

15 ### A.   Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents, patent applications, published applications and publications, Genbank sequences,
20 websites and other published materials referred to throughout the entire disclosure herein are, unless noted otherwise, incorporated by reference in their entirety. In the event that there are a plurality of definitions for terms herein, those in this section prevail.

As used herein, directed evolution refers to mehods that adapt"
25 natural proteins or protein domains to work in new chemical or biological environments and/or to elicit new functions. It is more a more broad-based technology than DNA shuffling.

As used herein, high-throughput screening (HTS) refers to processes that test a large number of samples, such as samples of test
30 proteins or cells containing nucleic acids encoding the proteins of interest to identify structures of interest or the identify test compounds that interact with the variant proteins or cells containing them. HTS operations are amenable to automation and are typically computerized to

handle sample preparation, assay procedures and the subsequent processing of large volumes of data.

As used herein, DNA shuffling is a PCR-based technology that produces random rearrangements between two or more sequence-related genes to generate related, although different, variants of given gene.

As used herein, "hits" are mutant proteins that have an alteration in any attribute, chemical, physical or biological property in which such alteration is sought. In the methods herein, hits are generally generated by systematically replacing each amino acid in a protein or a domain thereof with a selected amino acid, typically Alanine, Glycine, Serine or any amino acid, as long as each residue is replaced with the same residue. Hits may be generated by other methods known to those of skill in the art and tested by the high throughput methods herein. For purposes herein a Hit typically has activity with respect to the function of interest that differs by at least 10%, 20%, 30% or more from the wild type or native protein. The desired alteration, which is generally a reduction in activity, will depend upon the function or property of interest.

As used herein, "leads" are "hits" whose activity has been optimized for the particular attribute, chemical, physical or biological property. In the methods herein, leads are generally produced by systematically replacing the hit loci with all remaining 18 amino acids, and identifying those among the resulting proteins that have a desired activity. The leads may be further optimized by replacement of a plurality of "hit" residues. Leads may be generated by other methods known to those of skill in the and tested by the high throughput methods herein. For purposes herein a lead typically has activity with respect to the function of interest that differs from the native activity, by a desired amount and is at by at least 10%, 20%, 30% or more from the wild type or native protein. Generally a Lead will have an activity that is 2 to 10 or more times the native protein for the activity of interest. As with hits, the

change in the activity is dependent upon the activity that is "evolved." The desired alteration will depend upon the function or property of interest.

As used herein, MOI is multiplicity of infection.

As used herein, ip, with reference to a virus or recombinant vector, refers to a titer of infectious particles.

As used herein, pp refers to the total number of vector (or virus) physical particles

As used herein, biological and pharmacological activity includes any activity of a biological pharmaceutical agent and includes, but is not limited to, biological efficiency, transduction efficiency, gene/transgene expression, differential gene expression and induction activity, titer, progeny productivity, toxicity, cytotoxicity, immunogenicity, cell proliferation and/or differentiation activity, anti-viral activity, morphogenetic activity, teratogenetic activity, pathogenetic activity, therapeutic activity, tumor suppressor activity, ontogenetic activity, oncogenetic activity, enzymatic activity, pharmacological activity, cell/tissue tropism and delivery.

As used herein, "output signal" refers to parameters that can be followed over time and, if desired, quantified. For example, when a virus infects a cell, the infected cell undergoes a number of changes. Any such change that can be monitored and used to assess infection, is an "output signal," and the cell is referred to as a "reporter cell." Output signals include, but are not limited to, enzyme activity, fluorescence, luminescence, amount of product produced and other such signals. Output signals include expression of a viral gene or viral gene product, including heterologous genes (transgenes) inserted into the virus. Such expression is a function of time ("t") after infection, which in turn is related to the amount of virus used to infect the cell, and, hence, the concentration of virus ("s") in the infecting composition. For higher concentrations the output signal is higher. For any particular

concentration, the output signal increases as a function of time until a plateau is reached.   Output signals may also measure the interaction between cells, expressing heterologous genes, and biological agents

As used herein, adeno-associated virus (AAV) is a defective and

5 non-pathogenic parvovirus that requires co-infection with either adenovirus or herpes virus for its growth and multiplication, able of providing helper functions. A variety of serotypes are known, and contemplated herein. Such serotypes include, but are not limited to: AAV-1 (Genbank accession no. NC002077; accession no. VR-645); AAV-

10 2 (Genbank accession no. NC001401; accession no. VR-680); AAV-3 (Genbank accession no. NC001729; acession no. VR-681); AAV-3b (Genbank accession no. NC001863); AAV-4 (Genbank accession no. NC001829; ATCC accession no. VR-646); AAV-6 (Genbank accession no.NC001729); and avian associated adeno-virus (ATCC accession no.

15 VR-1449). The preparation and use of AAVs as vectors for gene expression *in vitro* and for *in vivo* use for gene therapy is well known (see, *e.g.*, U.S. Patent Nos. 4,797,368, 5,139,941, 5,798,390 and 6,127,175; Tessier *et al.* (2001) *J. Virol. 75:*375-383; Salvetti *et al.* (1998) *Hum Gene Ther 20:*695-706; Chadeuf *et al.* (2000) *J Gene Med*

20 *2:*260-268).

As used herein, the activity of a Rep protein or of a capsid protein refers to any biological activity that can be assessed. In particular, herein, the activity assessed for the rep proteins is the amount (*i.e.*, titer) of AAV produced by a cell.

25 As used herein, the Hill equation is a mathematical model that relates the concentration of a drug (*i.e.*, test compound or substance) to the response being measured

30 $$y = \frac{y_{max}[D]^x}{[D]^n + [D_{50}]^n} \quad ,$$

where y is the variable being measured, such as a response, signal, y_{max} is the maximal response achievable, [D] is the molar concentration of a drug, $[D_{50}]$ is the concentration that produces a 50% maximal response to the drug, n is the slope parameter, which is 1 if the drug binds to a single

5   site and with no cooperativity between or among sites. A Hill plot is $\log_{10}$ of the ratio of ligand-occupied receptor to free receptor vs. log [D] (M). The slope is n, where a slope of greater than 1 indicates cooperativity among binding sites, and a slope of less than 1 can indicate heterogeneity of binding.   This general equation has been employed for assessing

10   interactions in complex biological systems (see, published International PCT application No. WO 01/44809 based on PCT n° PCT/FR00/03503, see, also, EXAMPLES).

As used herein, in the Hill-based analysis (published International PCT application No. WO 01/44809 based on PCT n° PCT/FR00/03503),

15   the parameters, $\pi, \kappa, \tau, \epsilon, \eta, \theta$, are as follows:

π potency of the biological agent acting on the assay (cell-based) system;

κ constant of resistance of the assay system to elicit a response to a biological agent;

20   ϵ is global efficiency of the process or reaction triggered by the biological agent on the assay system;

τ is the apparent titer of the biological agent;

θ is the absolute titer of the biological agent; and

η is the heterogeneity of the biological process or reaction.

25   In particular, as used herein, the parameters π (potency) or κ (constant of resistance) are used to respectively assess the potency of a test agent to produce a response in an assay system and the resistance of the assay system to respond to the agent.

As used herein, ϵ(efficiency), is the slope at the inflexion point of

30   the Hill curve (or, in general, of any other sigmoidal or linear approximation), to asses the efficiency of the global reaction (the

biological agent and the assay system taken together) to elicit the biological or pharmacological response.

As used herein, τ (apparent titer) is used to measure the limiting dilution or the apparent titer of the biological agent.

5 As used herein, θ (absolute titer), is used to measure the absolute limiting dilution or titer of the biological agent.

As used herein, η (heterogeneity) measures the existence of discontinuous phases along the global reaction, which is reflected by an abrupt change in the value of the Hill coefficient or in the constant of

10 resistance.

As used herein, a library of mutants refers to a collection of plasmids or other vehicles that carrying (encoding) the gene variants, such that individual plasmid or other vehicles carry individual gene variants. When a library of proteins is contemplated, it will be so-stated.

15 As used herein, a "reporter cell" is the cell that "reports", *i.e.*, undergoes the change, in response to introduction of the nucleic acid infection and, therefore, it is named here a reporter cell.

As used herein, "reporter" or "reporter moiety" refers to any moiety that allows for the detection of a molecule of interest, such as a protein

20 expressed by a cell. Typical reporter moieties include, for example, fluorescent proteins, such as red, blue and green fluorescent proteins. For expression in cells, nucleic acid encoding the reporter moiety can be expressed as a fusion protein with a protein of interest or under to the control of a promoter of interest.

25 As used herein, a titering virus increases or decreases the output signal from a reporter virus, which is a virus that can be detected, such as by a detectable label or signal.

As used herein, phenotype refers to the physical or other manifestation of a genotype (a sequence of a gene). In the methods

30 herein, phenotypes that result from alteration of a genotype are assessed.

As used herein, activity refers to the function or property to be evolved An active site refers to a site(s) responsible or that participates in conferring the activity or function. The activity or active site evolved (the function or property and the site conferring or participating in

5   conferring the activity) may have nothing to do with natural activities of a protein. For example, it could be an 'active site' for conferring immunogenicity (immunogenic sites or epitopes) on a protein.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their known,

10   three-letter or one-letter abbreviations (see, Table 1). The nucleotides, which occur in the various nucleic acid fragments, are designated with the standard single-letter designations used routinely in the art.

As used herein, amino acid residue refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide

15   linkages. The amino acid residues described herein are presumed to be in the "L" isomeric form. Residues in the "D" isomeric form, which are so-designated, can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide.

20   COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in *J. Biol. Chem., 243*:3552-59 (1969) and adopted at 37 C.F.R. § § 1.821 - 1.822, abbreviations for amino acid residues are shown in the following Table:

25   **Table 1**
                              Table of Correspondence

SYMBOL		
1-Letter	**3-Letter**	**AMINO ACID**
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine

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SYMBOL		
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
X	Xaa	Unknown or other

It should be noted that all amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids, such as those referred to in 37 C.F.R. § § 1.821- 1.822, and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue

sequence indicates a peptide bond to a further sequence of one or more amino acid residues or to an amino-terminal group such as NH_2 or to a carboxyl-terminal group such as COOH.

In a peptide or protein, suitable conservative substitutions of amino

5 acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*,

10 4th Edition, 1987, The Benjamin/Cummings Pub. co., p.224).

Such substitutions are preferably made in accordance with those set forth in TABLE 2 as follows:

TABLE 2

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Pro (P)	Ala; Gly
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined

35 empirically or in accord with known conservative substitutions.

As used herein, nucleic acids include DNA, RNA and analogs thereof, including protein nucleic acids (PNA) and mixture thereof. Nucleic acids can be single or double stranded. When referring to probes or primers, optionally labeled, with a detectable label, such as a

fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that they are *statistically* unique of low copy number (typically less than 5, preferably less than 3) for probing or priming a library. Generally a probe or primer contains at

5 least 14, 16 or 30 contiguous of sequence complementary to or identical a gene of interest. Probes and primers can be 10, 14, 16, 20, 30, 50, 100 or more nucleic acid bases long.

As used herein, by homologous means about greater than 25% nucleic acid sequence identity, preferably 25% 40%, 60%, 80%, 90% or

10 95%. The intended percentage will be specified. The terms "homology" and "identity" are often used interchangeably. In general, sequences are aligned so that the highest order match is obtained (see, *e.g.*: *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*,

15 Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991;

20 Carillo *et al.* (1988) *SIAM J Applied Math 48*:1073). By sequence identity, the number of conserved amino acids are determined by standard alignment algorithms programs, and are used with default gap penalties established by each supplier. Substantially homologous nucleic acid molecules would hybridize typically at moderate stringency or at high

25 stringency all along the length of the nucleic acid of interest. Also contemplated are nucleic acid molecules that contain degenerate codons in place of codons in the hybridizing nucleic acid molecule.

As used herein, a nucleic acid homolog refers to a nucleic acid that includes a preselected conserved nucleotide sequence, such as a

30 sequence encoding a therapeutic polypeptide. By the term "substantially homologous" is meant having at least 80%, preferably at least 90%,

most preferably at least 95% homology therewith or a less percentage of homology or identity and conserved biological activity or function.

The terms "homology" and "identity" are often used interchangeably. In this regard, percent homology or identity may be

5   determined, for example, by comparing sequence information using a GAP computer program. The GAP program uses the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443 (1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482 (1981). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or

10   amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program may include: (1) a unitary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.*

15   14:6745 (1986), as described by Schwartz and Dayhoff, eds., *ATLAS OF PROTEIN SEQUENCE AND STRUCTURE*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

20   Whether any two nucleic acid molecules have nucleotide sequences that are, for example, at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% ,"identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85*:2444 (1988).

25   Alternatively the BLAST function of the National Center for Biotechnology Information database may be used to determine identity

In general, sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published techniques. (See, *e.g.*: *Computational*

30   *Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed.,

Academic Press, New York, 1993; *Computer Analysis of Sequence Data,*
Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey,
1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic
Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux,

5 J., eds., M Stockton Press, New York, 1991). While there exist a number
of methods to measure identity between two polynucleotide or
polypeptide sequences, the term "identity" is well known to skilled
artisans (Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073 (1988)).
Methods commonly employed to determine identity or similarity between

10 two sequences include, but are not limited to, those disclosed in Guide to
Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego,
1994, and Carillo, H. & Lipton, D., *SIAM J Applied Math 48*:1073
(1988). Methods to determine identity and similarity are codified in
computer programs. Preferred computer program methods to determine

15 identity and similarity between two sequences include, but are not limited
to, GCG program package (Devereux, J., *et al., Nucleic Acids Research*
12(I):387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F., *et al., J*
Molec Biol 215:403 (1990)), and CLUSTALW. For sequences displaying
a relatively high degree of homology, alignment can be effected manually

20 by simply lining up the sequences and manually or visually matching the
conserved portions.

Therefore, as used herein, the term "identity" represents a
comparison between a test and a reference polypeptide or polynucleotide.
For example, a test polypeptide may be defined as any polypeptide that

25 is 90% or more identical to a reference polypeptide.

For the alignments presented herein (see, Fig. 5) for the AAV
serotype, the CLUSTALW program was employed with parameters set as
follows: scoring matrix BLOSUM, gap open 10, gap extend 0.1, gap
distance 40% and transitions/transversions 0.5; specific residue penalties

30 for hydrophobic amino acids (DEGKNPQRS), distance between gaps for

which the penalties are augmented was 8, and gaps of extremities penalized less than internal gaps.

As used herein, a "corresponding" position on a protein, such as the AAV rep protein, refers to an amino acid position based upon

5   alignment to maximize sequence identity. For AAV Rep proteins an alignment of the Rep 78 protein from AAV-2 and the corresponding protein from other AAV serotypes (AAV-1, AAV-6, AAV-3, AAV-3B, AAV-4, AAV-2 and AAV-5) is shown in Figure 5. The "hit" positions are shown in italics.

10   As used herein, the term at least "90% identical to" refers to percent identities from 90 to 100% relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of 100 amino acids are compared. No more than 10% (i.e., 10 out of 100)

15   amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons may be made between a test and reference polynucleotides. Such differences may be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they may be clustered in one or more locations of varying

20   length up to the maximum allowable, e.g. 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, or deletions.

As used herein, it is also understood that the terms substantially identical or similar varies with the context as understood by those skilled

25   in the relevant art.

As used herein, genetic therapy involves the transfer of heterologous nucleic acids to the certain cells, target cells, of a mammal, particularly a human, with a disorder or conditions for which such therapy is sought. The nucleic acid, such as DNA, is introduced into the selected

30   target cells in a manner such that the heterologous nucleic acid, such as DNA, is expressed and a therapeutic product encoded thereby is

produced. Alternatively, the heterologous nucleic acid, such as DNA, may in some manner mediate expression of DNA that encodes the therapeutic product, or it may encode a product, such as a peptide or RNA that in some manner mediates, directly or indirectly, expression of a

5 therapeutic product. Genetic therapy may also be used to deliver nucleic acid encoding a gene product that replaces a defective gene or supplements a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid may encode a therapeutic compound, such as a growth factor inhibitor thereof, or a tumor necrosis

10 factor or inhibitor thereof, such as a receptor therefor, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous nucleic acid, such as DNA, encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to

15 enhance or otherwise alter the product or expression thereof. Genetic therapy may also involve delivery of an inhibitor or repressor or other modulator of gene expression.

As used herein, heterologous or foreign nucleic acid, such as DNA and RNA, are used interchangeably and refer to DNA or RNA that does

20 not occur naturally as part of the genome in which it is present or which is found in a location or locations in the genome that differ from that in which it occurs in nature. Heterologous nucleic acid is generally not endogenous to the cell into which it is introduced, but has been obtained from another cell or prepared synthetically. Generally, although not

25 necessarily, such nucleic acid encodes RNA and proteins that are not normally produced by the cell in which it is expressed. Any DNA or RNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed is herein encompassed by heterologous DNA. Heterologous DNA and RNA may also encode RNA or

30 proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes.

Examples of heterologous nucleic acid include, but are not limited to, nucleic acid that encodes traceable marker proteins, such as a protein that confers drug resistance, nucleic acid that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies.

5

Hence, herein heterologous DNA or foreign DNA, includes a DNA molecule not present in the exact orientation and position as the counterpart DNA molecule found in the genome. It may also refer to a DNA molecule from another organism or species (*i.e.*, exogenous).

10   As used herein, a therapeutically effective product introduced by genetic therapy is a product that is encoded by heterologous nucleic acid, typically DNA, that, upon introduction of the nucleic acid into a host, a product is expressed that ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures the

15   disease.

As used herein, isolated with reference to a nucleic acid molecule or polypeptide or other biomolecule means that the nucleic acid or polypeptide has separated from the genetic environment from which the polypeptide or nucleic acid were obtained. It may also mean altered from

20   the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell

25   is considered isolated. Also intended as an "isolated polypeptide" or an "isolated polynucleotide" are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source. For example, a recombinantly produced version of a compounds can be substantially purified by the one-step method

30   described in Smith and Johnson, *Gene 67:*31-40 (1988). The terms isolated and purified are sometimes used interchangeably.

Thus, by "isolated" is meant that the nucleic is free of the coding sequences of those genes that, in the naturally-occurring genome of the organism (if any) immediately flank the gene encoding the nucleic acid of interest. Isolated DNA may be single-stranded or double-stranded, and

5   may be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It may be identical to a native DNA sequence, or may differ from such sequence by the deletion, addition, or substitution of one or more nucleotides.

Isolated or purified as it refers to preparations made from biological

10   cells or hosts means any cell extract containing the indicated DNA or protein including a crude extract of the DNA or protein of interest. For example, in the case of a protein, a purified preparation can be obtained following an individual technique or a series of preparative or biochemical techniques and the DNA or protein of interest can be present at various

15   degrees of purity in these preparations. The procedures may include for example, but are not limited to, ammonium sulfate fractionation, gel filtration, ion exchange change chromatography, affinity chromatography, density gradient centrifugation and electrophoresis.

A preparation of DNA or protein that is "substantially pure" or

20   "isolated" should be understood to mean a preparation free from naturally occurring materials with which such DNA or protein is normally associated in nature. "Essentially pure" should be understood to mean a "highly" purified preparation that contains at least 95% of the DNA or protein of interest.

25   A cell extract that contains the DNA or protein of interest should be understood to mean a homogenate preparation or cell-free preparation obtained from cells that express the protein or contain the DNA of interest. The term "cell extract" is intended to include culture media, especially spent culture media from which the cells have been removed.

30   As used herein, receptor refers to a biologically active molecule that specifically binds to (or with) other molecules. The term "receptor

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protein" may be used to more specifically indicate the proteinaceous nature of a specific receptor.

As used herein, recombinant refers to any progeny formed as the result of genetic engineering.

5   As used herein, a promoter region refers to the portion of DNA of a gene that controls transcription of the DNA to which it is operatively linked.   The promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the

10   promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of the RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

15   As used herein, the phrase "operatively linked" generally means the sequences or segments have been covalently joined into one piece of DNA, whether in single or double stranded form, whereby control or regulatory sequences on one segment control or permit expression or replication or other such control of other segments. The two segments

20   are not necessarily contiguous. For gene expression a DNA sequence and a regulatory sequence(s) are connected in such a way to control or permit gene expression when the appropriate molecular, e.g., transcriptional activator proteins, are bound to the regulatory sequence(s).

As used herein, production by recombinant means by using

25   recombinant DNA methods means the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA, including cloning expression of genes and methods, such as gene shuffling and phage display with screening for desired specificities.

As used herein, a splice variant refers to a variant produced by

30   differential processing of a primary transcript of genomic DNA that results in more than one type of mRNA.

As used herein, a composition refers to any mixture of two or more products or compounds. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, a combination refers to any association between
5   two or more items.

As used herein, substantially identical to a product means sufficiently similar so that the property of interest is sufficiently unchanged so that the substantially identical product can be used in place of the product.

10   As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they
15   are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to
20   the chromosome. "Plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. Other such other forms of expression vectors that serve equivalent functions and that become known in the art subsequently hereto.

As used herein, vector is also used interchangeable with "virus
25   vector" or "viral vector". In this case, which will be clear from the context, the "vector" is not self-replicating. Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells.

As used herein, transduction refers to the process of gene transfer
30   and expression into mammalian and other cells mediated by viruses. Transfection refers to the process when mediated by plasmids.

As used herein, "polymorphism" refers to the coexistence of more than one form of a gene or portion thereof. A portion of a gene of which there are at least two different forms, i.e., two different nucleotide sequences, is referred to as a "polymorphic region of a gene". A

5   polymorphic region can be a single nucleotide, referred to as a single nucleotide polymorphism (SNP), the identity of which differs in different alleles. A polymorphic region can also be several nucleotides in length.

As used herein, "polymorphic gene" refers to a gene having at least one polymorphic region.

10   As used herein, "allele", which is used interchangeably herein with "allelic variant" refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different

15   alleles of a gene, the subject is said to be heterozygous for the gene. Alleles of a specific gene can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions, and insertions of nucleotides. An allele of a gene can also be a form of a gene containing a mutation.

20   As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid molecule comprising an open reading frame and including at least one exon and (optionally) an intron sequence. A gene can be either RNA or DNA. Genes may include regions preceding and following the coding region (leader and trailer).

25   As used herein, "intron" refers to a DNA sequence present in a given gene which is spliced out during mRNA maturation.

As used herein, "nucleotide sequence complementary to the nucleotide sequence set forth in SEQ ID NO: x" refers to the nucleotide sequence of the complementary strand of a nucleic acid strand having

30   SEQ ID NO: x. The term "complementary strand" is used herein interchangeably with the term "complement". The complement of a

nucleic acid strand can be the complement of a coding strand or the complement of a non-coding strand. When referring to double stranded nucleic acids, the complement of a nucleic acid having SEQ ID NO: x refers to the complementary strand of the strand having SEQ ID NO: x or

5   to any nucleic acid having the nucleotide sequence of the complementary strand of SEQ ID NO: x. When referring to a single stranded nucleic acid having the nucleotide sequence SEQ ID NO: x, the complement of this nucleic acid is a nucleic acid having a nucleotide sequence which is complementary to that of SEQ ID NO: x.

10   As used herein, the term "coding sequence" refers to that portion of a gene that encodes an amino acid sequence of a protein.

As used herein, the term "sense strand" refers to that strand of a double-stranded nucleic acid molecule that has the sequence of the mRNA that encodes the amino acid sequence encoded by the double-

15   stranded nucleic acid molecule.

As used herein, the term "antisense strand" refers to that strand of a double-stranded nucleic acid molecule that is the complement of the sequence of the mRNA that encodes the amino acid sequence encoded by the double-stranded nucleic acid molecule.

20   As used herein, an array refers to a collection of elements, such as nucleic acid molecules, containing three or more members. An addressable array is one in which the members of the array are identifiable, typically by position on a solid phase support or by virtue of an identifiable or detectable label, such as by color, fluorescence,

25   electronic signal (*i.e.* radiofrequency (RF), microwave or other frequency that does not substantially alter the interaction of the molecules of interest), bar code or other symbology, chemical or other such label. Hence, in general the members of the array are immobilized to discrete identifiable loci on the surface of a solid phase or directly or indirectly

30   linked to or otherwise associated with the identifiable label, such as

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affixed to a microsphere or other particulate support (herein referred to as beads) and suspended in solution or spread out on a surface.

As used herein, a support (also referred to as a matrix support, a matrix, an insoluble support or solid support) refers to any solid or

5   semisolid or insoluble support to which a molecule of interest, typically a biological molecule, organic molecule or biospecific ligand is linked or contacted. Such materials include any materials that are used as affinity matrices or supports for chemical and biological molecule syntheses and analyses, such as, but are not limited to: polystyrene, polycarbonate,

10   polypropylene, nylon, glass, dextran, chitin, sand, pumice, agarose, polysaccharides, dendrimers, buckyballs, polyacrylamide, silicon, rubber, and other materials used as supports for solid phase syntheses, affinity separations and purifications, hybridization reactions, immunoassays and other such applications. The matrix herein can be particulate or can be

15   in the form of a continuous surface, such as a microtiter dish or well, a glass slide, a silicon chip, a nitrocellulose sheet, nylon mesh, or other such materials. When particulate, typically the particles have at least one dimension in the 5-10 mm range or smaller. Such particles, referred collectively herein as "beads", are often, but not necessarily, spherical.

20   Such reference, however, does not constrain the geometry of the matrix, which may be any shape, including random shapes, needles, fibers, and elongated. Roughly spherical "beads", particularly microspheres that can be used in the liquid phase, are also contemplated. The "beads" may include additional components, such as magnetic or paramagnetic

25   particles (see, *e.g.*,, Dyna beads (Dynal, Oslo, Norway)) for separation using magnets, as long as the additional components do not interfere with the methods and analyses herein.

As used herein, matrix or support particles refers to matrix materials that are in the form of discrete particles. The particles have any

30   shape and dimensions, but typically have at least one dimension that is 100 mm or less, 50 mm or less, 10 mm or less, 1 mm or less, 100 μm or

less, 50 μm or less and typically have a size that is 100 mm^3 or less, 50 mm^3 or less, 10 mm^3 or less, and 1 mm^3 or less, 100 μm^3 or less and may be order of cubic microns. Such particles are collectively called "beads."

As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) *Biochem.* *11*:942-944).

B. High Throughput Process

Provided herein are high throughput process for the generation of and identification of proteins that exhibit desired phenotypes. The processes, include methods that are particularly adapted for high throughput protocols, which require accurate methods for identifying modified proteins.

A general directed evolution process includes the following steps:

1.   Generation of diversity at the nucleic acid level, on the gene to be 'evolved'

2.   phenotypic characterization of the gene variants generated; and

3.   identification of optimized gene variants.

The processes provided herein effect these steps that can be performed in a high throughput format (see, FIGURES 1) that is optionally automated. A distinguishing characteristic of the processes provided herein, is that each candidate nucleic acid molecule is separately generated and screened. In an automated process at least some of the steps are performed without human intervention and are generally controlled by software. Most, if not all steps, are performed in addressable formats, such as at discrete locations in or on solid supports, such as microtiter plates or in other addressable formats, such as linked to coded supports. The supports can be electronically, physically,

chemically or otherwise identifiable, such as by an identifiable symbology, including a bar code, or can be color coded.

1. Generation of Diversity using a semi-rational approach

A semi-rational approach to creating diversity or evolving genes is

5 provided herein. The goal is to create diversity but to decrease the number of molecules to be screened. By reducing the numbers, the molecules can be screened in high throughput format molecule-by-molecule (or groups thereof).

Generation of diversity at the nucleic level, in principle, can be

10 accomplished by a number of diverse technologies like mutagenesis (either site-directed or random), recombination, shuffling and de-novo synthesis. These different technologies differ in the degree of diversity they generate as well as in the minimal length of the unitary change they can introduce (from single base to large domains). The outcome of step 1

15 is a collection of diverse, although highly related, molecules that constitutes what is called a 'library'.

This step is crucial, since it provides the initial conditions for the entire process and is determinative of the outcome. The chances of finding an optimized gene version in a library is a function of the total

20 diversity present in the library. In addition, the type of diversity introduced (such as, but not limited to, single point mutations, multiple point mutations, scarce small rearrangements, recombination of large domains, multiple shuffling) condition the outcome, particularly with respect to the generation of new variants compared to the original gene,

25 and the probability that the new variants, not only exhibit the "evolved" function or property, but also work in their natural biological networks where they are expected to act by interacting, recognizing, and/or being recognized, by a large panoply of other proteins and other molecules.

Rapid discovery of protein variants at the amino acid level by rational mutagenesis (aa-scan)

A method, referred to herein as an amino-acid scan method for directed evolution, is provided herein for generating protein variants. This method can be performed on an entire protein or selected domains thereof, or can be used to identify benchmark sequences, such as functional domains, and, for example, recombine them as exchangeable units or restrict the diversity to limited or specific regions of the protein. Not only can this method be used with the processes provided herein, but it also has applications for any methods that use such variants or require generation of such variants, such as, but not limited to, searches for consensus sequences and homology regions that are used in functional genomics, functional proteomics; comparative modeling in protein crystallography and protein modeling; searches for natural diversity, (*e.g.*, directed evolution methods in 6,171,820, 6,238,884, 6,174,673, 6,057,103, 6,001,574, 5,763,239,); exon- or family-shuffling-based diversity (*e.g.*, directed evolution using gene shuffling (see, *e.g.*, U.S. Patent Nos. 6,096,548, 6,117,679, 6,165,793, 6,180,406, 6,132,970); the optimization of only the CDRs regions (*e.g.*, directed evolution of antibodies see., *e.g.*, U.S. Patent Nos. 5,723,323, 6,258,530, 5770,434, 5,862,514) and other methods (see, *e.g.*, U.S. Patent Nos. 5,837,500, 5,571,698, 6,156,509).

The amino-acid scanning-based method provided herein has advantages that prior methods do not have. For example, prior methods are based upon the underlying assumption that there are parts of the molecule (gene or protein) that are sufficiently adapted to perform their respective function, and further changes are not advantageous. Such methods do not look at total potential plasticity of a given molecule, but at the plasticity still permitted while keeping some basic functions in place. By choosing this route, however, additional potential variation is missed. The potential in the intrinsic plasticity of those regions that are presumed 'preserved' is lost. For instance, methods (*e.g.*, those in U.S.

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Patent Nos. 6,171,820, 6,238,884, 6,174,673, 6,057,103, 6,001,574, 5,763,239) that use natural diversity can miss the potential plasticity within those regions that are naturally 'conserved', i.e. there where there is no natural diversity. Methods that rely on exon- or family-shuffling-

5   based diversity can miss the potential plasticity within regions contained in the shuffled fragments, i.e. within the fragments exchanged as a block.

The method provided herein in contrast is sufficiently flexible to create mutants at a variety of levels, including at the single amino acid level; i.e. the method can generate mutants that differ from each other at

10   a single amino and not at a larger block level. The challenge solved by the method herein is to generate diversity at the single amino acid level, without moving too close to a pure 'random' approach, which results in an intractable number of mutants.

The method provided herein is based on the premise that there are

15   single amino acids or small blocks of sequence of amino acids that are either (1) directly involved in the activities that the methods 'evolve' (these amino acids would be at or close to the 'active sites' of the protein); or (2) directly involved in maintaining within the protein the intra-molecular environment that allows the active site(s) to stay active.

20   Potential plasticity at the amino acid level can be exploited if amino acids or blocks of amino acids directly involved in the active sites for the activity to be evolved are known. Often they are not known. The problem that is solved herein, however, is how to exploit the potential plasticity at the amino acid level when nothing is known about the

25   structure of the protein in question or about the position of its single or numerous active sites.

The technology referred to herein as amino acid-scanning has been used to precisely identify those amino acids directly involved in the active sites of some enzymes and receptors (see, *e.g.*, Becl-Sickinger *et al.*

30   (1994) *Eur. J. Biochem. 223*:947-958; Gibbs *et al.* (1991)*J. Biol. Chem. 266*:8923-8931; Matsushita *et al.* (2000) *J. Biol. Chem. 275*:11044-

11049) but has not been employed for directed evolution or for the generation of diversity. The amino acid scan as practiced in the prior art is used to produce a set of protein mutants, often within the region suspected to contain the active site(s), such that in each individual

5　mutant a selected residue, such as Ala, replaces a different amino acid. Ala or other neutral amino acids generally, although not necessarily, is selected as a replacement amino acid since, except in instances in which the replaced amino acid is directly involved in an active site, it should have a neutral effect on the protein activity and not disturb the native

10　secondary structure of the protein. In instances in which the replaced amino acid is directly involved in an active site the activity of that site will be lost or altered. Amino-acid scanning, such as Ala-scanning, has been successfully applied for the identification of active sites in a number of proteins, and has been performed in computer-based rational drug design

15　methods. Other amino acids, particularly amino acids that have a neutral effect, such as Glycine, can also be used.

　　　　The amino acid scanning method is employed herein for the generation of the mutant proteins for screening for identification of sites or loci in a target protein or regions in a protein that alter a selected

20　activity. In performing this method, the amino acids are each replaced, one-by-one along the full-length of the protein, or one-by-one in pre-selected domains, such as domains that possess a desired activity or exhibit a particular function. Once sites of interest are identified other methods for generating diversity from the resulting molecules can be

25　employed or the further steps of the method provided herein can be performed.

　　　　The method includes the following steps:

　　　　　　　　(1)　　Identification of the active site(s) on the full length protein sequence. In one embodiment a full-length amino acid-

30　scan, typically, although not necessarily, an Ala-scan, or the identification and positioning of the active site(s) on proteins of either known or

unknown function. For purposes herein, an active site is not necessarily the natural active sites involved in the natural activity of a target protein, but those amino acids involved in the activities of the proteins under 'directed evolution' with the purpose of either gain, improvement or loss

5   of function.

The whole process of the 'identification of the active site(s) on the full length protein sequence requires the following sub-steps:

a.   Generation of a mutant library (on the gene to be evolved) in which each individual mutant contains a single

10   mutation located at a different amino acid position and that includes a systematic replacement of the native amino acid by Ala or any other amino acid (always the same throughout the entire protein sequence);

b.   phenotypic characterization of the individual mutants, one-by-one and assessment of mutant protein activity;

15

c.   identification of those mutants that display an alteration, typically a decrease, in the selected protein activity, thus, indicating that amino acids directly involved in the active site(s) have been hit. The aa positions whose aa-scan mutations display an

20   alteration, typically a loss or decrease, in activity are named HITS.

The identification of the active site(s) (HITS) is thus, by this method, made in a completely unbiased manner. There are no assumptions about the specific structure of the protein in question nor any knowledge or assumptions about the active site(s). The results of the

25   amino acid scan identify such sites.

Once the active site(s) (the HITS) has(ve) been identified, those amino acids either at or surrounding the active sites, such as within 1, 2, 3, . . . 10, 20 or any selected regions, as the unitary elements of exchange and generate diversity either at or around one of those sites or

30   as a combined diversity at several sites at a time can be assessed. This process includes the following steps:

a.   Generation of a new mutant library (on the gene to be evolved) in which each individual mutant contains either single or multiple mutations located at (or surrounding) a specific active site (a HIT) position detected by the precedent aa-scan process. In the example these

5   mutations include replacement, in each individual mutant, of the native amino acid located either at (or surrounding) the HIT position by one of all other possible amino acids, such that, in the library, and at (or surrounding) each HIT position the native amino acid has been replaced by all possible amino acids.

10   b.   Identification of those mutants that display an increase in protein activity, thus indicating that a new sequence at or surrounding an active site has been identified with higher activity compared to the native sequence. These optimized sequences are named LEADS.

15   The process can be repeated as many times as desired, in search for new combinations of optimal amino acids at (or surrounding) the different HIT sites. Each time, the process includes the steps of generating of a new mutant library (of the gene to be evolved) in which each individual mutant contains either single or multiple mutations located

20   at (or surrounding) a specific active site (a HIT) position; phenotypic characterization of the individual mutants, one-by-one and assessment of mutant protein activity; and identification of those mutants that display an increase in protein activity, thus indicating that a new sequence at or surrounding an active site has been identified with higher activity

25   compared to the native sequence. These optimized sequences are again named LEADS (second generation LEADS).

2.   **Phenotypic characterization of the gene variants**

This step requires the expression of the gene variants in order to allow them to manifest their respective phenotypes. Gene expression can

30   be accomplished by different means: *in vitro*, in reconstituted systems or *in vivo* in cellular systems, including bacterial and eukaryotic cells. For all

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exemplification purposes, reference is made to *in vivo* systems. Those of skill in the art can readily adapt these methods for *in vitro* systems, including those using biochemical assays.

This step is a crucial step for several reasons:

5   (a)   **Expression system and protein processing.**

Depending on the system used (either bacteria or eukaryotic cells), as well as on the specific gene to be 'evolved', the variant proteins may or may not be appropriately processed, especially when post-translational modifications are involved, and therefore be able or not to elicit their

10 potential activity. Consequently, the expression system (bacteria vs. eukaryotic cells) has to be carefully chosen.

      (b)   **Standardization of the expression system.**

The technologies available for gene transfer and expression into either bacteria or eukaryotic (let's consider mammalian) cells widely vary

15 in their intrinsic efficiencies. While it is very easy to efficiently transfer and express genes in bacteria by chemical/physical methods (transformation), that is not the case for mammalian cells, where the transformation (here called transfection) process is inefficient and unreliable, specially when reproducibility and robustness are necessary in

20 miniature, large number- and small scale high throughput settings like those necessary to analyze gene variant libraries. Therefore, when transfection is used on mammalian cells, the specific activity measured for the individual variants in the library most probably does not accurately reflect the real specific activity of the molecules involved. As provided

25 herein, transduction, the process of gene transfer and expression into mammalian and other cells mediated by viruses, overcomes the limitations of transfection.

(3)   Characterization.

A distinction must be made between the 'expression' of the gene variants and their 'phenotypic characterization'. The expression system (either bacteria or mammalian cells) is only the vehicle to convert the

5   gene variants into protein variants. The phenotypic characterization is performed on the protein variants, and may have nothing to do, depending on the specific system under study, with the cellular system used to express the variants. The phenotypic characterization requires the use of specific assays (either biochemical (cell-free) or cell-based assays)

10   in which the activity of the different cell mutants is challenged and assessed. In addition to the implications discussed below, these assays must be designed in such a way that they reflect the final environment in which the 'evolved' protein is expected to act. As an example, when optimizing an enzyme to be used in an artificial industrial setting, the

15   assay should reproduce those conditions (temperature, pH, media composition...) of the real-life industrial reaction mixture, which may be relatively easy to do. When the final destination of the 'evolved' protein is a complex biological setting, such as the intracellular environment, the extra-cellular milieu (example: circulating proteins) or the structure of a

20   virus, the necessary assay(s) may be quite difficult to setup. With a few exceptions, most of the work done so far on directed evolution has been made on simple enzymes for which all the necessary settings are relatively easy to implement.

Methods for accurately titering viruses

25   Much progress in gene therapy, genomics, biotechnology and, in general, biomedical sciences, depends on the ability to generate and analyze large numbers and small amounts of specific viruses. High throughput technologies are employed in disciplines such as functional genomics and gene therapy in which the use of viruses plays a key role

30   for the efficient transfer and analysis of large collections of genes or libraries. Also, virus samples and biomedical samples containing viruses

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are routinely analyzed in thousands of hospitals, health centers, academic labs and biotech setting.

Furthermore in processes herein, accurate titration can be important in at least two steps in the process. After preparation of the viruses with

5   the mutated variant, and prior to screening, it is necessary to know the concentration of titer of the viruses in the sample, so that results among the samples can be compared. The methods in this section designated Real Time Virus Titering (RTVT") and (TREE) are advantageously used.

The methods in this section are also used in data analysis when

10   measuring the output signal. As described below, output signal can be assessed by a Hill analysis or a second order polynomial or other algorithm that describes the interaction of biological molecules in complex system. In addition, where the output signal is actually the number of viral particles or ip produced, the methods in this section RTVT and TREE

15   are advantageously used.

Prior art virus titration methods (RCA, dRA...),for determining the amount of virus present in a biological sample, are based on the assessment of some kind of output signal, such as cytopathic effect, lysis or plaques and cell fusion focuses, induced in a reporter cell following a

20   fixed time after infection with varying concentrations of the sample containing the virus. The lowest concentration of the sample at which no signal can be measured is taken as the titer of the virus in that sample. These approaches are known as "serial dilution" or "limiting dilution" methods. In limiting dilution methods, one single virus concentration,

25   measured at a given time end-point gives rise to a single measurement of the output signal. These methods tend to be destructive in that assessment destroys the reaction so that only a single measurement can be taken on a sample.

Real Time Virus Titering (RTVT™)

When a virus infects a cell, the infected cell undergoes a number
of changes that can be followed over time and quantified. Such changes
are designated herein as the "output signal". The cell reports an output

5   signal in response to the infection and, therefore, it is named here a
reporter cell. One such output signal, is, for example, the expression of
the genes carried by the virus (whether they are viral genes or
exogenous genes (transgenes)). The output signal (for instance the level
of expression of those genes) develops over time and depends, mainly,

10   on two factors: i) the time point ("t") at which its level is measured after
infection and ii) the amount of virus infecting the cell; i.e. the
concentration of the virus preparation used to infect the cell ("s").
The output signal, at a given time point after infection, will be higher for
higher concentrations of the virus infecting the reporter cells; and for any

15   given concentration of virus, the output signal increases with time after
infection until it generally reaches a plateau level.

Real Time Virus Titering (RTVT) published as International PCT
application No. WO 01/186291, which is based on PCT/FR01/01366 and
EXAMPLES below) uses non-destructive methods for the assessment of

20   output signal. Real Time Virus Titering is a viral titration method based on
the kinetic analysis of the development of the output signal in
virus-infected cells, tested at a single concentration of virus or biological
sample. Instead of fixing the time point after infection and varying the
concentration of the sample as is done in limiting dilution methods, in the

25   Real Time Virus Titerng RTVT™ method, a fixed concentration of virus is
used and the generation of a signal over time is assessed. Hence the
signal is measured as a function of time at a single virus concentration. In
this situation, a single virus sample (concentration), whose output signal
is measured at a number of time points, can give rise to as many

30   measurements of the output signal as needed, and, eventually to a
continuous, over time, assessment of the signal in real time.

Real Time Virus Titerng RTVT™ can be advantageously used in high throughput methods in which large numbers of biological samples are analyzed at the same time. This is the case, for instance, when titering viruses in a virus library. Limiting dilution methods rely on the output

5   signal generated by a number of dilutions of each individual sample. If, for example, 10 dilutions (or experimental points) of each virus are used for a titration using a limiting dilution method, the analysis of a library containing 10,000 viruses require analysis of 10^5 (*i.e.,* 10 x 10,000) experimental points. The Real Time Virus Titerng RTVT™ method requires

10   only one dilution per sample, thereby requiring 10-fold fewer experimental points than a limiting dilution method. For a Real Time Virus Titerng RTVT™ titering system, the time (tβ) necessary for the output signal to reach a reference value (β) is a direct function of the concentration of virus. Thus, tβ can be used to directly determine the concentration of the

15   virus.

A limitation of the Real Time Virus Titerng RTVT™ limiting dilution titering method, however, is that not all the viruses (nor the genes carried by the viruses) generate a readily measured output signal that can be followed over time using non-destructive methods.

20   **Tagged Replication and expression enhancement (TREE)**

A method for titering designated Tagged Replication and Expression Enhancement Technology (TREE™) is provided herein. This system includes: i) a cell, ii) a reporter virus carrying a reporter gene, whose

25   activity can be followed over time by a non- destructive method (*i.e.,* fluorescence), iii) the virus (or virus library to be titered), herein referred to as the "titering virus". The elements are employed such that the virus to be titered interferes with any output signal generated by the reporter virus, leading to either decrease or increase in the amount of that signal.

30   The higher the amount of virus to be titered, the higher is the interference with the reporter virus and output signal. In the absence of virus to be titered, the kinetics of the output signal generated by the reporter virus

are followed using the Real Time Virus Titerng RTVT™ titering method. In the presence of increasing amounts of the virus to be titered the output signal takes longer (or shorter) to develop as a function of the amount of virus to be titered.

5   Using the TREE™ titering method, $t\beta$, the time necessary for the output signal to reach a reference value (β) is a direct function of the concentration of virus and, therefore, $t\beta$ can be used to determine the concentration of the virus to be titered. It is demonstrated herein (see the EXAMPLES) that when using the TREE system for titering, once an

10   appropriate reference value (β) is determined for the output signal generated from the reporter virus, the time $t\beta$ is a function of the concentration of the virus being titered (see Example). Therefore, the concentration (titer) of the virus to be titered, can be assessed by assessing the change induced in $t\beta$ by an aliquot of the virus to be titered.

15   In a calibrated TREE titering assay, only one aliquot virus to be titered is needed to determine its titer, which is determined by measuring the shift in the $t\beta$ of the system. The only condition is that the virus to be titered must "interfere" (*i.e.*, increase or decrease) the output signal of the reporter virus.

20   A calibration curve representing $t\beta$ vs. the amount of virus to be titered is obtained using aliquots of a reference batch of virus of known titer (previously determined using any titering procedure). The calibration curve can then be used to determine the amount of virus in a sample of unknown titer, based on the change caused by an aliquot of the sample

25   on the $t\beta$ of the system and the corresponding titer read from the calibration curve.

3.   **Identification of gene variants**

There are at least two considerations in this step:

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(a) Selection vs. screening.

Depending on the specific protein involved, and under certain and very specific assay conditions, those variants that have been 'evolved' may elicit a selective advantage over the native version. This situation

5 represents the most simple case: the cells (bacteria or mammalian) expressing the library of protein variants, as a pool or mixture, can be simply exposed to the selective conditions which by themselves will allow to put in evidence the best optimized variants. This situation is however very rare and difficult to achieve. It can be hardly believed that for any

10 protein that one may want to optimize, a suitable 'selective' assay could be set up. For the vast majority of the cases, selection will not be possible. Therefore pools of molecules cannot be used, because the specific readouts of the assays could not be attributed to individual variants. When the simplistic selection approach is not possible, then two

15 things are absolutely needed: (a) a 'one-by-one' approach, i.e. each individual variant must be physically separated from the others and its activity tested independently; (b) an accurate and quantitative analysis that can distinguish slight differences in activity among the different variants along a wide range of performance values.

20 ### (b) Accurate quantitative analytics

When selection is not possible, the optimized variants must be distinguished from the native variant otherwise. The different degrees of optimization among the different variants in the library should, in addition, be distinguished if those variants showing the highest optimization level

25 are to be identified. A powerful quantitative analytical protocol is then mandatory. These analytics should be able to attribute quantitative features (on the activity tested in the specific assay) to each of the variants tested and to rank them according to their individual performance. This requires, in addition, that each variant in the library is

30 assayed individually; the use of pools or mixtures of molecules would hamper the ability to identify the right variants.

For such analysis, the output signal can be assessed by a Hill analysis (see Examples and (published International PCT application No. WO 01/44809 based on PCT n° PCT/FR00/03503),.) or a second order polynomial (see, Examples and (Drittanti *et al.* (2000) *Gene Ther.* 7: 924-

5   929)) or other algorithm that describes the interaction of biological molecules in complex system, such as the interaction between cells and biological agents. In addition, where the output signal is actually the number of viral particles or ip produced, the methods in this designated Real Time Virus Titering (RTVT™) and Tagged Replication and expression

10   enhancement (TREE™) are advantageously used (for a discussion of RTVT™, see, International PCT application No. PCT/FR01/01366 published as International PCT application No. WO 01/186291 and the EXAMPLES below) or a refinement of that method provide herein and designated Tagged Replication and expression enhancement (TREE™) described

15   above and in the examples.

C.   Practice of the process

In one embodiment, the process provided herein includes the following steps.

1.   Generation of diversity or source of existing diversity

20   Generation of a plasmid library containing the genetic variants. The genetic variants are physically separated from each other. Any model such as, but not limited to, amino acid scanning, mutagenesis, or recombination may be used to generate the plasmid library.

2.   Expression of the genetic variants

25   Any method for expression of variants is contemplated. In particular the following alternatives are particularly suitable for high throughput performance.

a. Expression in bacterial hosts

The mutated forms of the nucleic acid are prepared or introduced

30   into plasmids for expression in bacterial cells. The genetic variants are expressed from suitable bacterial cells, which are prepared by

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transformation aliquots of the cells with each member of the plasmid library (each genetic variant continues to be physically separated from each other).

b. Expression in eukaryotic host cells

5   A virus library is generated from the plasmid library. The virus library, in which each different member is separately maintained, is prepared by:

(1)   Transfection of the plasmid library into appropriate virus-producer cells (viruses produced, each one carrying a
10   different genetic variant present in the original plasmid library, are physically separated from each other);

(2)   Titration of the virus library (of each individual virus present in the library, separately). Titration is effected by any method, but generally by either a method designated Real Time Virus
15   Titering (RTVT™) (see, International PCT application No. PCT/FR01/01366 published as International PCT application No. WO 01/186291 and the EXAMPLES below) or a refinement of that method provide herein and designated Tagged Replication and expression enhancement (TREE™) described above and in the examples;

20   (3)   Standardization of the virus library to equal concentrations of all the individual viruses in the library (individual viruses continue to be physically separated from each other);

(4)   Expression of the genetic variants from appropriate mammalian cells by transduction with the virus library (each
25   genetic variant continues to be physically separated from each other and each individual virus is handled separately from the others).

3.   Phenotypic characterization of the variant proteins.

The variant proteins are expressed (from either plasmids in bacterial cells (step 2) or viruses in mammalian cells (step 4)) and their activity is
30   assessed in one or more appropriate specific assays. The assays can be both types: biochemical (cell-free) assays and/or cell-based assays. The

variant proteins in the library are physically separated from each other and their activity is individually assessed on a one-by-one basis.

The assays can be performed in one of a variety of ways, including, but are not limited to:

5   a.   Using a single-point dilution for each individual variant protein, followed by a kinetic analysis (multiple time points) of the read-out by technologies like Tagged Replication and expression enhancement (TREE™), or any other appropriate technology

        b.   Using serial dilutions of each individual variant protein,
10   followed by, for example, the Hill-based analysis of the read-out by technologies or any other appropriate technology. Hill based analyses assess the interaction between cells and biological agents (see, published International PCT application No. WO 01/44809 based on PCT n° PCT/FR00/03503).

15   The goal of these methods is to identify proteins having an evolved function or property.

Lead identification

Based on the results obtained from the assays described above, each individual protein variant is individually tested for the parameters
20   that assess the activity, property, function or structure of interest. Variants are ranked out according to their activity features. Those variant proteins best suited for the specificities of each individual project and system under study are then selected. The selected leads can be used for the desired purpose or further evolved or mutated to achieve desired
25   activities.

Typically, as for most directed evolution methods, the process is an iterative one, in which mutated variants are produced, screened, the best identified and then selected. The selected variants are then subjected to further evolution and the screening process repeated. This is repeated
30   until the desired goal is achieved.

This further evolution may employ the methods herein or any directed evolution method or combinations thereof. The methods for variant production will include the amino-acid scan method herein, which provides a rational approach to variant generation. Other rounds can

5   include combinations of any other method for directed evolution known and/or combinations thereof.

D.   Directed evolution of a viral gene

Recombinant viruses have been developed for use as gene therapy vectors. Gene therapy applications are hampered by the need for

10   development of vectors with traits optimized for this application. The high throughput methods provided herein are ideally suited for development of such vectors. In addition to use for development of recombinant viral vectors for gene therapy, these methods can also be used to study and modify the viral vector backbone architecture, trans-

15   complementing helper functions, where appropriate, regulatable and tissue specific promoters and transgene and genomic sequence analyses. Recombinant AAV (rAAV) is a gene therapy vector that can serve as a model for application of the methods herein for these and other purposes.

Adeno-associated virus (AAV) is a defective and non-pathogenic

20   parvovirus that requires co-infection with either adenovirus or herpes virus, which provide helper functions, for its growth and multiplication. There is an extensive body of knowledge regarding AAV biology and genetics (see, *e.g.*, Weitzman *et al.* (1996) *J. Virol. 70*: 2240-2248 (1996); Walker *et al.* (1997) *J. Virol. 71*:2722-2730; Urabe *et al.* (1999)

25   *J. Virol. 23*:2682-2693; Davis *et al.* (2000) *J. Virol. 23*:74:2936-2942; Yoon *et al.* (2001) *J. Virol. 75*:3230-3239; Deng *et al.* (1992) *Anal Biochem 200*:81-85; Drittanti *et al.* (2000) *Gene Therapy 7*:924-929; Srivastava *et al.* (1983) *J. Virol. 45*:555-564; Hermonat *et al.* (1984) *J. Virol. 51*:329-339; Chejanovsky *et al.* (1989) *Virology 173*:120-128;

30   Chejanovsky *et al.* (1990) *J. Virol. 64*:1764-1770; Owens *et al.* (1991) *Virology 184*:14-22; Owens *et al.* (1992) *J. Virol. 66*:1236-1240;

Qicheng Yang *et al.* (1992) *J. Virol.66*:6058-6069; Qicheng Yang *et al.* (1993) *J. Virol. 67*:4442-4447; Owens *et al.* (1993) *J. Virol. 62*:997-1005; Sirkka *et al.* (1994) *J. Virol. 68*:2947-2957; Ramesh *et al.* (1995) *Biochem. Biophy. Res. Com.* Vol 210 (3), 717-725; Sirkka (1995) *J.*

5 *Virol. 69*:6787-6796; Sirkka *et al.* (1996) *Biochem. Biophy. Res. Com. 220*:294-299; Ryan *et al.* (1996) *J. Virol. 70*:1542-1553; Weitzman *et al.* (1996) *J. Virol. 70*:2440-2448; Walker *et al.* (1997) *J. Virol. 71*:2722-2730; Walker *et al.* (1997) *J. Virol. 71*:6996-7004; Davis *et al.* (1999) *J. Virol. 73*:2084-2093; Urabe *et al.* (1999) *J. Virol.73*:2682-2693; Gavin

10 *et al.* (1999) *J. Virol. 73*:9433-9445; Davis *et al.* (2000) *J. Virol. 74*:2936-2942; Pei Wu *et al.* (2000) *J. Virol. 74*:8635-8647; Alessandro Marcello *et al.* (2000) *J. Virol.74*:9090-9098). AAV are members of the family *Parvoviridae* and are assigned to the genus *Dependovirus*. Members of this genus are small, non-enveloped, icosahedral with linear

15 and single-stranded DNA genomes, and have been isolated from many species ranging from insects to humans.

AAV can either remain latent after integration into host chromatin or replicate following infection. Without co-infection, AAV can enter host cells and preferentially integrate at a specific site on the *q* arm of

20 chromosome 19 in the human genome.

The AAV genome contains 4975 nucleotides and the coding sequence is flanked by two inverted terminal repeats (ITRs) on either side that are the only sequences in *cis* required for viral assembly and replication. The ITRs contain palindromic sequences, which form a hairpin

25 secondary structure, containing the viral origins of replication. The ITRs are organized in three segments: the Rep binding site (RBS), the terminal resolution site (TRS), and a spacer region separating the RBS from the TRS.

Regulation of AAV genes is complex and involves positive and

30 negative regulation of viral transcription. For example, the regulatory proteins Rep 78 and Rep 68 interact with viral promoters to establish a

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feedback loop (Beaton *et al.* (1989) *J. Virol 63*:4450-4454; Hermonat (1994) *Cancer Lett 81*:129-136). Expression from the p5 and p19 promoters is negatively regulated in *trans* by these proteins. Rep 78 and 68, which are required for this regulation, have bind to inverted terminal

5 repeats (ITRs; Ashktorab *et al.* (1989) *J. Virol. 63*:3034-3039) in a site- and stand-specific manner, *in vivo* and *in vitro*. This binding to ITRs induces a cleavage at the TRS and permits the replication of the hairpin structure, thus, illustrating the Rep helicase and endonuclease activities (Im *et al.* (1990) *Cell 61*:447-457; and Walker *et al.* (1997) *J. Virol.*

10 *71*:6996-7004), and the role of these non-structural proteins in the initial steps of DNA replication (Hermonat *et al.* (1984) *J. Virol. 52*:329-339). Rep 52 and 40, the two minor forms of the Rep proteins, do not bind to ITRs and are dispensable for viral DNA replication and site-specific integration (Im *et al.* (1992) *J. Virol. 66*:1119-112834; Ni *et al.* (1994) *J.*

15 *Virol. 68*:1128-1138.

The genome (see, FIG. 4) is organized into two open reading frames (ORFs, designated left and right) that encode structural capsid proteins (Cap) and non-structural proteins (Rep). There are three promoters: p5 (from nucleotides 255 to 261: TATTTAA), p19 (from

20 nucleotide 843 to 849: TATTTAA) and p40 (from nucleotides 1822 to 1827: ATATAA). The right-side ORF (see FIG. 4) encodes three capsid structural proteins (Vp 1-3). These three proteins, which are encoded by overlapping DNA, result from differential splicing and the use of an unusual initiator codon (Cassinoti *et al.* (1988) *Virology 167*:176-184).

25 Expression of the capsid genes is regulated by the p40 promoter. Capsid proteins VP1, VP2 and VP3 intiate from the p40 promoter. VP1 uses an alternate splice acceptor at nucleotide 2201; whereas VP2 and VP3 are derived from the same transcription unit, but VP2 use an ACG triplet as an initiation codon upstream from the start of VP3. On the left side of

30 the genome, two promoters p5 and p19 direct expression of four regulatory proteins. The left flanking sequence also uses a differential

splicing mechanism (Mendelson *et al.* (1986) *J. Virol 60*:823-832) to encode the Rep proteins, designated Rep 78, 68, 52 and 40 on the basis molecular weight. Rep 78 and 68 are translated from a transcript produced from the p5 promoter and are produced from the unspliced and

5 spliced form, respectively, of the transcript. Rep 52 and 40 are the translation products of unspliced and spliced transcripts from the p19 promoter.

The rep protein is a adeno-associated virus protein involved in a number of biological processes necessary to AAV replication. The

10 production of the rRep proteins enables viral DNA to replicate, encapsulate and integrate (McCarty *et al.* (1992) *J. Virol 66*:4050-4057; Horer *et al.* (1995) *J. Virol 69*:5485-5496, Berns *et al.* (1996) Biology of Adeno-associated virus, in Adeno-associated virus (AAV) Vectors in Gene Therapy, K.I. Berns and C. Giraud, Springer (1996); and Chiorini *et al.*

15 (1996) The Roles of AAV Rep Proteins in gene Expression and Targeted Integration, *from* Adeno-associated virus (AAV) Vectors in Gene Therapy, K.I. Berns and C. Giraud, Springer (1996)). A rep protein with improved activity could lead to increased amounts of virus progeny thus allowing higher productivity of rAAV vectors.

20 AAV and rAAV have many applications, including use as a gene transfer vector, for introducing heterologous nucleic acid into cells and for genetic therapy. Advances in the production of high-titer rAAV stocks to the transition to human clinical trials have been made, but improvement of rAAV production will be complemented with special attention to clinical

25 applications of rAAV vectors as successful gene therapy approach. Productivity of rAAV (i.e. the amount of vector particles that can be obtained per unitary manufacturing operation) is one of the rate limiting steps in the further development of rAAV as gene therapy vector. Methods for high throughput production and screening of rAAV have

30 been developed (see, *e.g.*, Drittanti *et al.* (2000) *Gene Therapy 7*:924-929) Briefly, as with the other steps in methods provided herein, the

plasmid preparation, transfection, virus productivity and titer and biological activity assessment are intended to be performed in automatable high throughput format, such as in a 96 well (or other number or multiples thereof, such as 384, 1536 . . . 9600, 9984 . . .)

5   formats.

Since the Rep protein is involved in replication it can serve as a target for increasing viral production. Since it has a variety of functions and its role in replication is complex, it has heretofore been difficult to identify mutations that result in increase viral production. The methods

10   herein, which rely on *in vivo* screening methods, permit optimization of its activities as assessed by increases in viral production. Provided herein are Rep proteins and viruses and viral vectors containing the mutated Rep proteins that provide such increase. The amino acid positions on the rep proteins that are relevant for rep proteins activities in terms of AAV or

15   rAAV virus production are provided. Those amino acid position are such that a change in the amino acid leads to a change in protein activity either to lower activity or increase activity. As shown herein, the alanine or amino acid scan revealed the amino acid positions important for such activity (i.e. hits). Subsequent mutations produced by systematically

20   replacing the amino acids at the hit positions with the remaining 18 amino acids produced so-called "leads" that have amino acid changes and result in higher virus production. In this particular example, the method used included the following specific steps.

Amino acid scan

25   In order to first identify those amino acid (aa) positions on the rep protein that are involved in rep protein activity, an Ala-scan was performed on the rep sequence. For this, each aa in the rep protein sequence was individually changed to Alanine. Each resulting mutant rep protein was then expressed and the amount of virus it could produced

30   measured as indicated below. The relative activity of each individual mutant compared to the native protein is indicated in FIG 3A. HITS are

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those mutants that produce a decrease in the activity of the protein (in the example: all the mutants with activities below about 20 % of the native activity).

In a second experimental round, which included a new set of
5   mutations and phenotypic analysis, each amino acid position hit by the Ala-scan step, was mutated by amino acid replacement of the native amino acid by the remaining 18 amino acids, using site directed-mutagenesis.

In both rounds, each mutant was individually designed, generated
10   and processed separately, and optionally in parallel with the other mutants. Neither combinatorial generation of mutants nor mixtures thereof were used in any step of the method.

A plasmid library was thus generated in which each plasmid contained a different mutant bearing a different amino acid at a different hit
15   position. Again, each resulting mutant rep protein was then expressed and the amount of virus it could produced measure as indicated below. The relative activity of each individual mutant compared to the native protein is indicated in FIGURE 3B. LEADS are those mutants that lead to an increase in the activity of the protein (in the example: the ten mutants
20   with activities higher, typically between 6 to 10 times, than the native activity).

Expression of the genetic variants and phenotypic characterization.

The rep protein acts as an intracellular protein through complex interaction with a molecular network composed by cellular proteins, DNA,
25   AAV proteins and adenoviral proteins (note: some adenovirus proteins have to be present for the rep protein to work). The final outcome of the rep protein activity is the virus offspring composed by infectious rAAV particles. It can be expected that the activity of rep mutants would affect the titer of the rAAV virus coming out of the cells.
30   As the phenotypic characterization of the rep variants can only be accomplished by assaying its activity from inside mammalian cells, a

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mammalian cell-based expression system as well as a mammalian cell-based assay was used. The individual rep protein variants were expressed in human 293 HEK cells, by transfection of the individual plasmids constituting the diverse plasmid library. All necessary functions were

5 provided as follows:

(a) the cellular proteins present in the permissive specific 293 HEK cells;

(b) the AAV necessary proteins and DNA were provided by co-transfection of the AAV cap gene as well as a rAAV plasmid vector

10 providing the necessary signaling and substrate ITRs sequences;

(c) the adenovirus (AV) proteins were provided by co-transfection with a plasmid expressing all the AV helper functions.

A library of recombinant viruses with mutant rep encoding genes was generated. Each recombinant, upon introduction into a mammalian

15 cell and expression resulted in production of rAAV infectious particles. The number of infectious particles produced by each recombinant was determined in order to assess the activity of the rep variant that had generated that amount of infectious particles.

The number of infectious particles produced was determined in a

20 cell-based assay in which the activity of a reporter gene, in the exemplified embodiment, the bacterial lacZ gene, or virus replication (Real time PCR) was performed to quantitatively assess the number of viruses. The limiting dilution (titer) for each virus preparation (each coming from a different rep variant) was determined by serial dilution of the viruses

25 produced, followed by infection of appropriate cells (293 HEK or HeLa rep/cap 32 cells) with each dilution for each virus and then by measurement of the activity of the reporter gene for each dilution of each virus. Hill plots (NAUTSCAN™) as described herein (published as International PCT application No. WO 01/44809 based on PCT n°

30 PCT/FR00/03503, Dec, 2000; see EXAMPLES) or a second order polynomial function (Drittanti *et al.* (2000) *Gene Ther.* 7: 924-929) was

used to analyze the readout data and to calculate the virus titers. Briefly, the titer was calculated from the second order polynomial function by non-linear regression fitting of the experimental data. The point where the polynomial curve reaches its minimum is considered to be the titer of

5 the rAAV preparation. A computer program for calculation of titers has been developed (see Drittanti *et al.* (2000) *Gene Ther. 7*: 924-929) to assess the minima.

The TREE method described herein can be used to analyze the readout data and to calculate the virus titers. The results are shown in the

10 EXAMPLE below.

Comparison between results of full-length Hit position analysis reporter here and the literature

The experiments identified a number of heretofore unknown mutation loci, which include the hits at positions: 4, 20, 22, 28, 32, 38,

15 39, 54, 59, 124, 125, 127, 132, 140, 161, 163, 193, 196, 197, 221, 228, 231, 234, 258, 260, 263, 264, 334, 335, 341, 342, 347, 350, 354, 363, 364, 367, 370, 376, 381, 389, 407, 411, 414, 420, 421, 422, 428, 429, 438, 440, 451, 460, 462, 484, 488, 495, 497, 498, 499, 503, 511, 512, 516, 517 and 518 with reference to the amino

20 acids in Rep78 and Rep 68. Rep 78 is encoded by nucleotides 321-2,186; Rep 68 is encoded by nucleotides 321-1906 and 2228-2252; Rep 52 is encoded by nucleotides 993-2186, and Rep 40 is encoded by amino acids 993-1906 and 2228-2252 of wildtype AAV.

Also among these are mutations that may have multiple effects.

25 Since the Rep coding region is quite complex, some of the mutations may have several effects. Amino acids 542, 598, 600 and 601, which are in the Rep 68 and 40 intron region, are also in the coding region of Rep 78 and 52. Codon 630 is in the coding region of Rep 68 and 40 and non coding region of Rep 78 and 52.

30 Mutations at 10, 86, 101, 334 and 519 have been previously identified, and mutations, at loci 64, 74, 88, 175, 237, 250 and 429, but with different amino acid substitutions, have been previously reported. In

all instances, however, the known mutations reportedly decrease the activity of Rep proteins. Among mutations described herein, are mutations that result in increases in the activity the Rep function as assessed by detecting increased AAV production.

5 **Lead identification.**

Based on the results obtained from the assays described herein (i.e. titer of virus produced by each rep variant), each individual rep variant was assigned a specific activity. Those variant proteins displaying the highest titers were selected as leads and are used to produce rAAV.

10 In further steps, rAAV and Rep proteins that contain a plurality of mutations based on the hits (see Table in the EXAMPLES, listing the hits and lead sites), are produced to produce rAAV and Rep proteins that have activity that is further optimized. Examples of such proteins and AAV containing such proteins are described in the EXAMPLES.

15 The rAAV rep mutants are used as expression vectors, which, for example, can be used transiently for the production of recombinant AAV stocks. Alternatively, the recombinant plasmids may be used to generate stable packaging cell lines. To create a stable producer cell line, the recombinant vectors expressing the AAV with mutant rep genes, for

20 example, are cotransfected into host cells with a plasmid expressing the neomycin phosphotransferase gene (neor) by transfection methods well known to those skilled in the art, followed by selection for G418 resistance.

Also among the uses of rAAV, particularly the high titer stocks

25 produced herein, is gene therapy for the purpose of transferring genetic information into appropriate host cells for the management and correction of human diseases including inherited and acquired disorders such as cancer and AIDS. The rAAV can be administered to a patient at therapeutically effective doses. A therapeutically effective dose refers to

30 that amount of the compound sufficient to result in amelioration of symptoms of disease.

Gene therapy

Toxicity and therapeutic efficacy of the rAAV can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LDS_{50} (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Doses that exhibit large therapeutic indices are preferred. Doses that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets rAAV to the site of treatment in order to minimize damage to untreated cells and reduce side effects.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such rAAV lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. A therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (ie., the concentration of the test compound which achieves a half-maximal infection or a half- maximal inhibition) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention. The specific

methods exemplified can be practiced with other species. The examples are intended to exemplify generic processes.

EXAMPLE 1

Titering or assessment of concentration by a method designated Real
5   **Time Vector Titering (RTVT™)**

This Example is based on the method described in International PCT application No. PCT/FR01/01366, based on French application n° 0005852, filed 9 May 2000, and published as International PCT application No. WO 01/186291. This method assesses the

10   titer or concentration of a biological agent (virus, gene transfer vector) in a sample, by measurement of the kinetics of change of a reporter parameter following the exposure of cells to the biological agent.

As noted above, reporter parameters may include, but are not limited to,: gene / transgene expression related to the gene/transgene

15   products, such as enzymatic activity, fluorescence, luminescence, antigen activity, binding to receptors or antibodies, and regulation of gene expression), differential gene expression, viral/vector progeny productivity, toxicity, cytotoxicity, cell proliferation and/or differentiation activity, anti-viral activity, morphogenetic activity, pathogenetic activity,

20   therapeutic activity, tumor suppressor activity, oncogenetic activity, pharmacological activity.

Serial dilution methods

The assessment of the concentration or titer of biological agents using current approaches needs for serial dilutions of the agent.

25   Serial dilutions of the agent are applied to a cell-based reported system, that elicits an output signal in response to the exposure to the agent. The intensity of the signal is a function of the concentration of the agent. The titer or concentration of the agent is determined as the highest dilution that still elicits a measurable response in the output. The higher the

30   number of dilutions tested, the higher the accuracy of the value obtained for the titer.

This approach requires a set of serial dilutions for every biological agent whose titer needs to be determined. Thus, the application of this approach to the simultaneous titration of a large number of different biological agents is limited by the number of experimental points needed

5 (example: for 30 biological samples: *20* serial dilutions x *30* biological agents: 600 experimental points).

The approach in International PCT application No. PCT/FR01/01366 published as International PCT application No. WO 01/186291

10 The intensity of the output signal (after exposure of reporter cells to the biological agent) is not only dependent on the *concentration of the* agent but also on the time after exposure. As time increases, the intensity of the signal increases. The kinetics of change of the intensity *over time* depends upon the concentration of the agent. Thus, lower concentrations

15 of the agent will require longer times for the intensity to reach a given value that would be reached in shorter times after exposure to higher concentrations of the same agent.

This approach (designated Real Time Virus Titering (RTVT™) uses the following: a reference plot representing the relationship between *the*

20 concentration of the agent and the time necessary for the intensity to reach a given threshold value is obtained using a reference preparation of biological agent, whose concentration or titer is known. This plot is then used to obtain the concentration of the biological agent under study by entering the time that a dilution of that agent needed for the intensity to

25 reach the threshold value.

Using this approach, there is no need for serial dilutions of the biological agent(s) under study. Once the reference plot (t_β vs c) is obtained, it can be used for the determination of the concentration or titer of any number of biological agents. Only one dilution of the biological

30 agent under study is necessary to obtain the corresponding value of t_β that is then used to obtain the concentration or titer using the reference plot.

Thus, the application of this approach to the simultaneous titration of large numbers of different biological agents is facilitated by the fact that only one dilution of each sample is needed (example: for 30 biological samples: *1* dilution x *30* biological agents: 30 experimental

5 points (compared to 600 needed with the current approach).

This approach is specially suited for the high throughput assessment of concentration or titer of large numbers of biological agents.

The system

The system includes the following elements:

10 a preparation of the biological agent (virus, gene transfer vector, protein,...) whose concentration or titer is unknown and has to be determined.

a reporter system including culture of a cell line (or a mixture of cell lines) that reacts to the exposure to the biological

15 agent by displaying a specific output signal.

a master preparation of a reference biological agent, of known concentration or titer, that is able to generate the output signal when the reporter cells are exposed to it.

Practice of the method

20 When the reporter cells are exposed to either the biological agent under study or the reference biological agent, an output signal is generated, that can be measured.

The intensity of the output signal is called i; the concentration of the biological agent used is called c, the time of exposure of the cells to the

25 biological agent is called t. The intensity of the output signal (i) is a function of c and t :

i increases as the concentration (c) of the biological agent applied to the cells increases;

i increases as the time of exposure of the cells to the biological

30 agent (t) increases.

If the time t after exposure of the cells to the biological agent is kept constant, then, i will change as a direct function of c .

If the concentration c of the biological agent is kept constant, then, i will be a direct function of t.

5   β is defined here as a threshold value of the intensity of the output signal, arbitrarily defined for every system under study.

$t\beta$ is defined here as the time necessary to reach the threshold β.

Use of β and $t\beta$ to determine the concentration or titer of a biological agent.

10   The reporter cells are exposed to serial dilutions of a reference biological agent, whose concentration (or titer) is previously known. The intensity of the output signal (i) is measured at several time points (t) for every concentration (serial dilutions) c of the reference biological agent.

i is plotted vs t , and that, for every concentration c used of the

15   reference biological agent.

Using the plots obtained above, and for every concentration c of the reference biological agent, the time $(t\beta)$ necessary for the intensity of the output signal to reach a threshold value β is obtained.

With the data obtained above, $t\beta$ is plotted vs c.

20   This plot represents the time necessary for the intensity of the output signal to reach the threshold value β as a function of the concentration of the biological agent used. T his is a standard plot and will be used to determine the unknown concentration of the biological agent under study by measuring the time that a given dilution of it needs

25   to give an output signal whose intensity equals the threshold β.

The reporter cells are exposed to a dilution of the biological agent under study (whose concentration or titer is to be determined). The intensity of the output signal (i) is measured over time until it reaches the threshold value β. The time necessary for i to reach the value β is

30   recorded as $t\beta$.

The $t\beta$ value recorded above is entered into the standard plot obtained above) and the corresponding c value is obtained.

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This *c* value represents the concentration or titer of the biological agent under study.

Example of the Real Time Virus Titerng RTVT™ titering method

Rat-2 cells were infected with serial dilutions of a reference preparation of a retroviral vector carrying the green fluorescent protein (GFP) gene (vector pSI-EGFPI see, Ropp *et al.* (1995) *Cytometry 21*:309-317). At increasing times after infection, the level of expression of the transgene was determined (as the level of fluorescence due to the GFP gene) as the output signal.

Table 3 represents the values obtained:

Concentration $(1 = 10^6$ particles/ml)	Time after infection (hrs)	Output signal fluorescence
0.1	16	20.4
	24	30.1
	40	95.1
	48	138.7
	64	157.3
0.25	16	26.8
	24	48.5
	40	173.3
	48	228.2
	64	191.7
0.5	16	38.1
	24	72
	40	198.7
	48	296.2
	64	203.7

The threshold value of $\beta = 100$ was arbitrarily selected for this example. The time $(t\beta)$ necessary for the output signal to reach the threshold β , for every concentration is shown in table 4.

Table 4

Concentration $(1 = 10^6$ particles/ml)	$t\beta$ (hrs)
0.1	42
0.25	31

A plot of tβ versus concentration for the reference virus shows that the concentration and tβ exhibit a clearly defined relationship, that allows for the calculation of the concentration (c) of a sample, if the corresponding tβ of that sample is known.

5
EXAMPLE 2

Tagged Replication and Expression Enhancement (TREE) for titering

As discussed above, TREE is a method for titering and standardization of preparations of viruses, vectors, antibodies, libraries, proteins, genes and any other moiety that is detectable based upon a
10 output signal, such as fluorescence. The TREE method is an improvement of the Real Time Virus Titering (RTVT) method (see, International PCT application No. PCT/FR01/01366 published as International PCT application No. WO 01/186291). It is performed a reporter moiety, such as a reporter virus (with a known titer) and the test sample (with
15 unknown titer). The reporter, such as a reporter virus has a readily detectable output signal that can be measured as a function of time. The effect of the moiety, such as a virus, of unknown titer is assessed. The moiety whose titer is assessed either increases or decreases the output signal as a function of time. This change in signal is used to assess the
20 amount or concentration of the moiety of unknown concentration, and hence its titer.

The method is exemplified herein using an AAV system for the determination of the titer of an AAV vector and an AAV-reporter vector as a competitor and wild type Adenovirus as helper virus. One of skill in
25 the art readily can adapt the method to other systems, including other viruses, and other moieties for which a reporter system can be developed. Other such moieties include, but are not limited to, viral vectors, plasmids, libraries, proteins, antibodies, vaccines, genes, and nucleic acid molecules.

Materials and Methods

1. Cells and Viruses

HeLa rep-cap32 cells, a HeLa derived cell line (kindly provided by P. Moullier, Laboratoire de Thérapie Génique, CHU, Nantes; see, Salvetti *et al.* (1998) *Hum Gene Ther 20*:695-706; Chadeuf *et al.* (2000) *J Gene Med 2*:260-268) was grown in DMEM with 10% fetal calf serum. These cells were plated 24 h before infection at a density of 1 x 10^4 cells in single well of 96-well plates. rAAV-LacZ (10^{10} ip/ml), rAAV-GFP (10^9 ip/ml) vectors and Human Adenovirus type 5 (Ad5) (10^{11} pfu/ml) were from CHU, Nantes.

Hela rep-cap32 cells had been produced by cotransfecting plasmid pspRC, which harbors the AAV *rep-cap* genome with the ITRs deleted (bp 190 to 4484 of wild-type AAV), with plasmid PGK-Neo, conferring resistance to G418 on Hela cells (see, Chadeuf *et al.* (2000) *J. Gene Med. 2*:260-268 and Salvetti *et al.* (1998(*Hum Gene Ther. 9*:695-706). Hela rep-cap 32 cells are a packaging line that harbor one copy of the genome with the ITRs deleted (see, also Tessier *et al.* (2001) *J. Virol. 75:*375-383).

Plasmid pspRC contains the AAV genome (positions 190-4,484 bp) with the ITRs deleted and was obtained by excising the *rep-cap* fragment (*Xba*I fragment) from the well-known vector psub201 (Samulski *et al.* (1987) *J Virol 61*:3096-3101; also called pSSV9) by *Xba*I digestion and inserting it into the *Xba*I site of plasmid pSP72 (Promega). Plasmid psub201 (see, *e.g.*, U.S. Patent No. 5,753,500) is a modified full-length AAV type 2 genomic clone contains all of the AAV type 2 wild-type coding regions and cis acting terminal repeats.

2. Infection and measurement

Four serial dilutions of a rAAV-LacZ (0.01, 0.0075, 0.005 and 0.0025 μl, see Table 2 below, designated samples 1-4, respectively) were made and used for co-infection of HeLa rep-cap32 cells together with 8 different Ad5 multiplicity of infection (MOI; from 0.1 to 100/cell) and with

10^{-3} ml (10^6 infectious particles (ip)) or 10^{-4} ml (10^5 ip) rAAV-GFP viral vector. All the samples were done in triplicate. After infection, the plates were read at different times, from 34.5 h to 80 h (every 30 minutes).

rAAV-GFP is an SSV9-derived vector; SSV9 is a clone containing the entire adeno-associated virus (AAV) genome inserted into the PvuII site of plasmid pEMBL (see, Du *et al.* (1996) *Gene Ther 3*:254-261). The rAAV-GFP and rAAV-LacZ plasmids are SSV9 with a GFP or LacZ gene under control of the cytomegalovirus (CMV) immediate-early promoter. All the samples were done in triplicate.

3. Process

Figure 2 shows the overall procedure in 96 well format. Cells were plated 24 h before infection. Co-infection of rAAV-GFP with serial dilutions of rAAV-LacZ together with Ad5 (different MOI), were done. Then the plates were read at different times using the Analyst AD&HT micro plate-reader (LJL BioSystems).

4. Analysis

For this kinetic technique, Fluorescence Intensity (FI) of the infected cells is measured as a function of the time. Serial dilutions of the AAV-competitor vector AAV-lacZ vector, which decreases the fluorescence signal, are performed. For this example, fluorescence was measured for AAV-GFP with 10^6 ip and 10^5 ip and then 10^6 ip of the AAV-GFP reporter virus in the serial dilutions of the competitor virus, AAV-lacZ vector in a 96-well format (samples 1-4, see Table below). Measurements were taken of each well and curves of FI (of the GFP) versus time (hrs) were obtained (see Figure 2B).

An arbitrary one value for FI (see Fig. 2B, 6×10^6 FI units), typically, though not necessarily, near the greatest separation among the curves so that the numbers are readily discernable, was selected. The point at which each of the curves intersect this value is beta time (tβ) for that combination of amounts of reporter plus dilution of the virus of unknown titer. tβ, taken from the FI vs. Time (hrs) curves, for each

sample containing a dilution of the unknown plus 10^6 ip of the reporter virus is set forth in column 2 of Table 2 below.

To determine the titer of the test virus, the $t\beta$ for the AAV GFP (reporter virus) is plotted versus quantity of ip (i.e a straight line between the $t\beta$ for the 10^6 ip and the 10^5 ip) (Fig. 2C). For any $t\beta$ of the unknown virus, the quantity of ip can be determined from this curve. The beta time ($t\beta$) of each sample (in this case for the different dilutions of rAAV LacZ mixed with 10^6 infectious particles of rAAV-GFP) is determined, and then the residual number of infectious particles of rAAV-GFP for each sample. The difference between 10^6 ip of rAAV-GFP put in each sample and the number of ip detected by fluorescence in the same well is the actual quantity of rAAV-GFP competed (consumed) by the unknown rAAV (in this case rAAV-LacZ). This number is determined for each dilution. The quantity rAAV-GFP consumed is the same quantity of unknown rAAV in the sample. This quantity is present in one volume of unknown rAAV, which in this example is 1 ml. Based upon this, the infectious titer of the unknown rAAV is determined. The results are shown in Table 2.

TABLE 2
AAV LacZ titration by TREE titration

Sample	volume (μl)	$t\beta$ (hrs)	Residual AAV-GFP	Consumed AAV-GFP	AAV LacZ Concentration (i.p./10^{-2} μl)
1	10.0×10^{-3}	66.5	5.56×10^5	4.44×10^5	4.44×10^5
2	7.5×10^{-3}	66.5	5.56×10^5	4.44×10^5	5.93×10^5
3	5.0×10^{-3}	65	7.06×10^5	2.94×10^5	5.88×10^5
4	2.5×10^{-3}	64	8.06×10^5	1.94×10^5	7.76×10^5

The average titer using this method was 6.01×10^{10} ip/ml (6.01×10^7 ip/μl = 6.01×10^5 ip/0.01 μl). The standard deviation was 1.37×10^{10} ip/ml with an error of $\pm 23\%$.

EXAMPLE 3

Hill Analysis of the screening assay output

It is important to have reliable methods for screening and/or evaluating the performance of a set of biological agents, such as a library

5 of viral or non-viral recombinant vectors, vaccines, recombinant proteins and antibodies, in a complex biological system, such as living target cells When developing such agents, for example gene therapy vectors and other agents for therapeutic use, it is necessary to be able to evaluate and compare performance among candidates.

10 The progress of gene transfer into gene therapy depends upon the capacity to develop gene transfer vectors into therapeutic drugs. Clinically relevant vectors need to be efficient and safe, in reaching and infecting target cells and in ensuring a persistent level of expression of the therapeutic gene with a minimum of adverse effects. The availability of

15 standardized quantitative methods, suitable for an accurate and objective assessment of titer, performance and safety, is necessary for the pharmaceutical development of gene vectors as drugs.

Any method for assessment is contemplated herein as long as it is adapted for use in a high throughput format. Of particular interest is the

20 Hill equation based method of International PCT application No.
WO 01/44809 (International PCT application No. PCT/FR00/03503, based on French application FR 9915884).

Two widely used parameters that provide quantitative information about the potential performance of a gene transfer vector preparation are

25 the titer of physical particles and the titer of infectious particles. Vector preparations with high titer of infectious particles and low physical particles/infectious particles ratio are considered to be of higher quality.

The titer in physical particles(pp) (see, *e.g.*, Mittereder *et al.* (1996) *J. Virol.* 70:7498-7509; Atkinson *et al.* (1998) *Nucl. Acids Res.* 26:2821-

30 2823; Kechli *et al.* (1998) *Hum. Gene Ther.* 9:587-590; and Nelson *et al.* (1998) *Hum. Gene Ther.* 9:2401-2405), which represents the total

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number of vector particles, is usually evaluated from the vector content by detecting the nucleic acid contents (nucleic acids hybridization and OD_{260} respectively for AAV and AdV), detecting viral protein content (for example, reverse transcriptase (RT) activity and p24 content for MLV and

5   HIV, respectively).

Among the physical particles (pp), there are particles potentially active in performing transduction (ip, infectious particles), as well as particles that are inactive (nip, non-infectious particles) (Ruffing *et al.* (1994) *J.Gen.Virol.* *75*:3385-3392; Kechli *et al.* (1998) *Hum. Gene Ther.*

10 *9*:587-590.). The pp and the ip/nip ratio, are features of the packaging system, the manufacturing process and the vector itself.

The infectious particles (ip) (infectious units, transducing units, etc.) are evidenced by the changes observed in the infected cells (vector DNA replication, provirus integration, cell lysis, transgene expression and

15 other observable parameters. Infectious particles (ip) measures the number of particles effective in performing a process whose output is being measured; not all particles participate or are capable of participating in all processes.

The precise assessment of ip is not straightforward. Existing

20 methods are mainly based on serial dilution experiments followed by either linear extrapolation or asymptote approximates. The titer of infectious particles (*ip*: infectious unity, transduction unity) (see, *e.g.*, Mittereder *et al.* (1996) *J. Virol.* *70*:7498-7509; Weitzman *et al.* (1996) *J. Virol.70*:1845-1854; Salvetti *et al.* (1998) *Hum. Gene Ther.* *9*:695-

25 706) is evaluated by the studying observed changes in infected cells, such as viral replication, provirus integration, cellular lysis and transgene expression, using methods based on serial dilutions, followed either by a linear extrapolation or an asymptotic approximation. Thus, *ip* measures the number of active particles in the measured process; it includes

30 physical particle (*pp*) and inactive particles (*nip* or *non-infectious particles*).

In order to resolve the problem of the titer determination and the comparison of different recombinant viruses used in gene therapy, the variation of the particles/infectious power ratio has been used (see, *e.g.,* Atkinson *et al.* (1998) *Nucl. Acids Res.* 26:2821-2823; and International

5   PCT application No. WO 99/11764, which describe a method that uses step of amplification viral genetic material in a host cellular line, preparation of vectors of unknown titer obtained by serial dilution and an internal check of known titer). In particular, the method uses cells infected with a viral preparation in the different wells of a microtiter plate,

10   viral genome replication in the host cells, nucleic acid hybridization and determination of the relative amount of replicated viral nucleic acid in each well.

All of these methods measure the physical particles (*pp*) titer and/or measure infectious particles (*ip*) titer in order to evaluate a gene transfer

15   vector. A high quality vector preparation is one with an high titer of infectious particles and a low *pp/ip* ratio. These parameters provide quantitative information on the performance of a gene transfer vector. Because of the inaccuracy of the procedures used for assessing pp and especially ip, these parameters are not informative enough to precisely

20   define the features of a gene therapy vector nor those of a particular preparation thereof. The actual procedures used for *pp* and *ip* evaluation change with the vector type, are not very reproducible nor exact, so these parameters do not contain enough information to allow a very fine definition of vector characteristics and performances.

25   **Hill equation-based analyses**

In this method complex biological processes, including those involving the response of cells (in vitro and in vivo) to biological agents, such as, for example, cells, viruses, vaccines, viral and non-viral gene vectors, antibodies, antigens, proteins in general and plasmids, are

30   characterized using the formal analysis first introduced by Hill (see, Hill (1910) *J. Physiol. 40*:4P; Hill (1913) *I. Biochem.J. 7*:471-480).

International PCT application No. WO 01/44809 (based on PCT/FR00/03503, priority claimed to French application FR 9915884) describes the use of the Hill equation (see, Hill (1910) *J. Physiol. 40*:4P; Hill (1913) *I. Biochem.J. 7*:471-480; see, International PCT application

5   No. PCT/FR00/03503) for analysis and characterization of the biological and/or pharmacological activity of biological agents (viruses, vectors or cells) on biological assay systems *in vitro* (cell-based) or *in vivo*.

A number of useful parameters, derived from the Hill equation, are scored and used to quantify relevant features of the biological agent, of

10   the cells, as well as of the biological process or reaction involved.

In particular, methods for calculation and analysis of the parameters of biological and pharmacological activity of native, attenuated, modified or recombinant viruses, vaccines, recombinant viral and non-viral gene transfer vectors, cells, antibodies and protein factors

15   in in vitro (cell-based) or in vivo assays are described. This method is adapted for high throughput processes and is sufficiently accurate to allow a very fine definition of vector characteristics and performances.

International PCT application No. WO 01/44809 provides, a standard process for evaluating the interaction between any biological

20   agent, such as a gene therapy vector, with a complex biological system (living target cells). It provides a screening process for a pool of complex biological agents, in order to select test agents that have a desired property, activity, structure or whatever is being sought.

Different biological agents and assay systems (cells) are compared

25   and ranked out on the basis of their performance, assessed through the Hill parameters. Thus, the accurate analysis and comparison of the biological response of complex assay systems (in vitro and in vivo) to complex biological agents is achieved experimentally. The Hill-based analysis ($\pi,\kappa,\tau,\epsilon,\eta,\theta$) is used for a variety of purposes, including, but not

30   limited to:

i) validation and optimization of the manufacturing processes used to obtain the biological agents;

ii) development and optimization of the components of the biological agents (proteins, genomes, genetic units);

5 iii) development and optimization of assays and analytical tests for the characterization of the biological agents.

The method includes the steps of:

(a) preparation, for each biological agent, of a sample scale, obtained by a serial dilution of the biological agent at a R1 concentration,

10 (b) incubation of each sample of the dilution scale obtained in 1, with the target cells at a constant concentration R2,

(c) determination of the P product from the reaction R1 + R2, at a t moment, in each the sample; and

(d) realization of a theoretical curve H from the experimental points

15 R1 and P, for each biological agent by iterative approximation of parameters of the reaction R1 + R2 → P, at the t moment, in accordance with this equation:

$$P = P_{max} \, (\pi R1)^r \, / \, (\kappa + (\pi R1)^r) \qquad r = 1,...,n \qquad (2)$$

in which:

20 R1 represents the biological agent concentration in a sample from the scale;

R2 is concentration of target cells (*in vitro* or *in vivo*)

P (output) represents the product from the reaction R1 + R2 at a t moment;

25 P_{max} represents the reaction maximal capacity;

κ represents, at a constant R2 concentration, the resistance of the biological system for responding to the biological agent (resistance constant R2);

r represents a coefficient that depends on R1 and

30 corresponds to the Hill coefficient; and

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π represents the intrinsic power of the R1 biological agent to induce a response in the biological system (P production at the t moment), and

(e) sorting the κ and π values obtained in (d) for each

5 biological agent and the biological agent, and then ranking according to the values thereof.

Using the parameters $(\pi,\kappa,\tau,\theta,\epsilon,\eta)$ the activity of a biological agent on a complex biological system, as well as its intrinsic features can be fully characterized and compared. In addition, different biological

10 systems either *in vitro* cell-based) or *in vivo* can be compared.

Hill Equation

The Hill equation:

$$P = \sum_{r=1}^{r=n} P_{max} \cdot R1^r / (K + (R1)^r) \qquad \text{R2 constant} \qquad (1)$$

15

where R1, P, P_{max} and K represent, respectively, the concentration of the reagent R1, the concentration of the product, the maximal capacity of the reaction and the 'affinity' constant between R1 and R2. The Hill's coefficient (r) is a function of R1. The coefficient r is equal to 1 when

20 independent non-interactive binding sites are involved between R1 and R2, such as in reactions that follow kinetics described by Michaelis-Menten; and r varies from 1 to n for systems where the sites involved in the interaction between the R1 and R2 are not independent from each other, and the affinity for R1 at any R2 binding site varies as a function of

25 either i) the degree of occupancy of other R2 sites; ii) the concentration of R1 itself or iii) the concentration of other (positive or negative) regulators.

The Hill equation, thus, is a general formalization that describes the interaction reaction between molecules. It expresses the amount of

30 product formed as a function of the concentration of the reagents and of the affinity constant of the system. Originally developed for the study of the dissociation between haemoglobin and oxygen, the Hill equation

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covers the formal Michaelis-Menten analysis of enzyme kinetics, the analysis of ligand-receptor binding and of the allosteric protein systems.

According to Hill, for a simple reaction like

$$R1 + R2 \rightarrow P$$

5 $\qquad K$

where the affinity between R1 and R2 changes with concentration of each, the Hill equation describes the accumulation of the product P as a function of the concentration of one of the reagents (R1) and of the intrinsic properties (K) of the system.

10 This equation can be applied to complex biological systems. For example, the response of the cells to infection (P), can be analyzed by applying an Hill-type equation. The amount of cells growing in vitro (R2) are infected with increasing concentrations of recombinant viruses (R1), and (P) is monitored. A Hill equation is iteratively fitted to the

15 experimental data.

For analyses of viral output as exemplified herein,

$$\longleftarrow \underline{\qquad} K \underline{\qquad} \longrightarrow$$
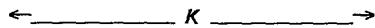
virus + cell → transduced cell → output (viral genome replication),
Equation (1) is specifically reformulated as:

20 $\qquad P = P_{max} (\pi R1)^r / (\kappa + (\pi R1)^r) \qquad\qquad r = 1,...,n \qquad\qquad (2)$

where P, P_{max}, R1, π, r and κ , as described above, represent, respectively, the output signal (P) (the level of viral gene expression, or the level of virus replication), the maximal output signal (Pmax), the initial concentration (R1) of infectious viral particles (those susceptible to trigger

25 the process leading to P), the potency of the vector (π; a factor that affects the concentration of the vector (R1) by its specific strength or activity, the Hill's coefficient (r) and the constant of resistance of the reaction or process (κ).

The constant of resistance κ

The concept of κ is analogous to those of dissociation, kinetics, equilibrium or affinity constants concepts for simple chemical and biological reactions. κ is a feature of the process (reaction) and of the

5   biological system tested (cell type). κ is a key parameter for the characterization of the assay system and the assessment of its performance as a test for the reaction under study.

κ measures the internal resistance offered by the process or reaction triggered by the biological agent, to proceed to P. κ is specific to

10   a particular process or reaction tested. In addition κ is specific to the particular biological system tested. Different cell lines and types will display different κ for the same reaction. Moreover, factors affecting the performance of a cell to accomplish the reaction (like contaminants, toxic agents, etc.) affect κ in that cell.

15   Variations in κ affect equation (2) by shifting the curve to the right or to the left, according to whether the value of κ increases or decreases, respectively. All curves differing only in κ are parallel each other.

κ finds its direct and practical application in i) assay development and validation and ii) assessment of the susceptibility or sensitivity of different

20   cell types or tissues to undertake the reaction under study and to be affected by it.

The potency π

π measures the intrinsic potency of the biological agent to accomplish P against the resistance (κ) offered by the reaction process.

25   For every infectious virus particle (R1) added to the assay, the actual activity of the virus added is given by πR1. In order to report an output P, the potency π has to push forward the reaction inside the cell against κ. π is specific to the particular biological agent for the reaction under study. π is a feature of the biological agent.

Different versions or variants of the biological agent will display different π for the same reaction. Thus, mutations, conformational changes or other modifications on the biological agent are expected to change its π for a given reaction process.

5   The concept of π is analogous to that of chemical activity by opposite to concentration for simple compounds. π is a correction factor that affects the concentration (R1) of the biological agent to indicate its actual strength or activity for a given reaction process.

Variations in π affect equation (2) by shifting the curve to the right

10   or to the left, according to whether the value of π decreases or increases, respectively. Curves differing only in π are not parallel each other. The slope of the curve given by equation (2) increases as π increases.

π is a key parameter for the characterization of the biological agent and the assessment of its performance to accomplish the reaction under

15   study. π finds its direct and practical application in i) biological agent optimization and development as it allows to compare the relative potency of variants of the agent.

π is a valuable tool in the field of vaccine, gene transfer vector and antibody development, for the comparison between two or more different

20   agents or different versions of the same agent, for performance. Two agents, for instance, may elicit equivalent potencies for gene transfer, while their potencies for immunogenicity be different. The use of π, a quantitative and accurate parameter for assessing potency, will allow for ranking the candidates according to their potency (*i.e.*, for gene transfer,

25   gene expression, immunogenicity and other such properties and activities) and to make rational decisions about the relative value of the agent leads.

The efficiency ϵ

ϵ measures the maximal global efficiency of the reaction process when a biological agent characterized by a given π value interacts with a

30   biological system characterized by a given κ. ϵ is specific to the particular couple biological agent (π) / biological system (κ) for the reaction under

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study. ϵ is a feature of the global reaction process and intervening reagents. Changes in either π, κ, or both, will lead to changes in ϵ.

The efficiency of the reaction process described by equation (2) is given by the increase in the output P that can be obtained by increasing

5 the input R1. Thus, the first derivative of P with respect to R1, or the slope of the curve described by equation (2), gives the global efficiency of the reaction at every R1 input. The maximal global efficiency, or ϵ, is given directly by either the slope at the inflection point of the curve described by equation (2) or by the maximum of its derivative $\delta P/\delta R1$.

10 The slope of the curve given by equation (2) and the maximum of $\delta P/\delta R1$ increase as ϵ increases.

ϵ is a key parameter for the characterization of the efficiency global process, considering the assay conditions and reagents all together. It is therefore useful for assay optimization once π and κ have been fixed and

15 to detect changes in π when κ is kept constant or, inversely, changes in κ while π is kept unchanged.

The heterogeneity index η

η measures the internal heterogeneity of the reaction process under study. Complex processes include a huge chain of individual and causally

20 events inside a multidimensional network of interrelated and interregulated biological reactions. Thus, the constant of resistance (κ) for the particular reaction process under study is a macroscopic indicator of the global resistance of that process ($\kappa = a1\kappa1 + a2\kappa2 + ...an\kappa n/n$). If the contribution of the individual microscopic constants of resistance (a1κ1,

25 a2κ2, ...anκn) for the individual steps involved in the process were homogeneous and no thresholds were present from one step to the next, then , no discontinuities in the increase of the Hill coefficient (i.e. in the change of κ) with R1 should be observed. The existence of a major heterogeneity among the κi values corresponding to the microscopic

30 individual steps (i.e. the existence of thresholds for the intermediate steps) might lead to a macroscopic discontinuity in the system.

Heterogeneity would cause a change in the rate of variation of the Hill coefficient and, which would require a jump in the macroscopic value of κ in order for equation (2) to fit the data.

The presence of internal heterogeneity in the reaction process can

5   be detected by the appearance of steps in the rate of change of the Hill coefficient, corresponding to the Hill curve that fits the experimental data. η is defined as an index of heterogeneity and its value corresponds to the number of steps in the rate of variation of the Hill coefficient (one step, $\eta = 1$; two steps, $\eta = 2$; n steps, $\eta = $ n).

10 η is a key parameter for the dissection and detailed analysis of the reaction process. It is useful for the independent optimization and development of every one of the steps identified by η.

As mentioned, the presence of steps in the rate of change of η translates in an abrupt discontinuity in κ. Therefore, every step is

15 determined by a different macroscopic constant of resistance κ. Systems with $\eta = 2$, can thus be described by a Hill equation in which κ takes two different values ($\kappa 1$ and $\kappa 2$), according to the R1 interval considered. One part of the curve is described by $\kappa 1$ and the other by $\kappa 2$.

Hill curves describing reaction processes characterized by $\eta = 2$, are

20 hybrids generated from two parallel Hill curves differing only in κ. The transition from one curve to the other may alter the smooth change in the slope of the resulting Hill curve.

The apparent titer τ

In the Hill equation (2), when R1 increases, r increases from 1 to

25 2,3,4... and P approaches its P_{max} value. On the other direction, on the contrary, R1 can only decrease up to a minimal point ($R1_{min}$), at which r and P reach their minimal values. The Hill sigmoidal curve is not symmetric, only the right arm is asymptotic (towards P_{max}). On the left arm, the curve has an origin at $R1_{min}$; the empirical curve does not fit the

30 data for values below $R1_{min}$.

From a biological point of view, the fact that P does not exist for R1 below $R1_{min}$, means that there is no 'product' when the concentration of 'substrate' is lower than $R1_{min}$; e.g. that the system is not responsive to concentrations below $R1_{min}$. The minimal concentration of R1 that the system can detect and report is $R1_{min}$.

5

In terms of biological agents, $R1_{min}$ represents the minimal amount that can elicit a response in a given reporter system, and it is represented by τ. The titer defined this way, is neither an asymptote value nor a value approached by extrapolation, but a precise parameter of the Hill equation,

10 at the very mathematical origin of the curve.

τ measures the limiting dilution or apparent titer of the biological reagent. The value of τ is determined by the limit of sensitivity of the biological assay system and of method used for the measurement of the product P; that is why it is said to be *apparent* titer.

15

τ is specific to the batch or stock of the biological reagent tested. τ represents the apparent concentration of the biological agent and is expressed in units per volume, e.g. the maximal dilution of the biological agent that leads to the production of P. τ is given by the maximal R1 for which the Hill coefficient reaches its minimal value (the Hill coefficient

20 becomes constant at a value equal or close to 1). The concept of τ corresponds to that of titer, of general use for viruses, antibodies and vectors. Variations in τ affect equation (2) by shifting the curve to the right or to the left, according to whether the value of τ decreases or increases, respectively.

25

τ is a key parameter that measures the 'apparent' concentration of a stock of the biological agent, which is necessary for whatever use it will be given.

The absolute titer θ

θ is a the parameter that measures the absolute concentration (titer) of a stock or batch of the biological agent. The value of θ is not determined by nor dependent on the limit of sensitivity of the biological

5   assay system or of the method used for the measurement of the product P; that is why it is said to be absolute titer. θ is specific to the batch or stock of the biological reagent tested. It represents the real physical concentration of the biological agent and is expressed in units per volume, e.g. the maximal dilution of the biological agent that leads to the

10   production of P.

θ is given by the following equation

$$\theta\pi = \tau/s \qquad (3),$$

where s is the sensitivity of the detection method. Therefore, for agents detected using the same method, the following expression is valid:

15   $$\theta_1\pi_1 /\tau_1 = \theta_2\pi_2 /\tau_2 = \theta_n\pi_n/\tau_n = \text{constant} \qquad (4)$$

Using equation (4), the ratio of the absolute titer θ, corresponding to two biological agent preparations, can be obtained from their respective π and τ. Variations in θ affect the equation (2) by shifting the curve to the right or to the left and/or by changing its slope.

20   **Compensation between π and κ**

π and κ may appear to compensate to generate two different Hill curves (one differing in π and the other one differing in κ) that would apparently fit with the same experimental data. As π and κ have opposite effects, two Hill curves; in which the increase in π is compensated by the

25   decrease in κ, and vice versa, may seem to represent the same curve, which could make it difficult to determine whether two Hill curves are different because a change in π or in κ.

Detailed analysis of the Hill curves indicates that π and κ do not compensate very well. Although curves differing in compensatory values

30   of either π or κ may vary close each other, they do not fit exactly in any of the two regions of highest curvature (before and after the inflection

point). This dispersion is caused by the fact that π, but not κ, changes the slope at the inflection point of the Hill curve. Therefore, ϵ, which is the slope of the Hill curve at the inflection point, can be used to easily differentiate between two Hill curves that apparently compensate for π

5 and κ.

Conclusions

The application of the Hill analysis to resolve complex biological processes is effective for the precise and objective understanding of processes like virus or vaccine action, entry, genome replication,

10 transgene expression, vector/transgene immunogenicity, cytotoxicity and other such parameters. The analysis is independent of the virus vaccine, vector and protein type involved and from the output parameter and variable measured, such as the internalized vector DNA, transgene mRNA level and transgene product activity.

15 As in the field of chemical pharmaceuticals, the structure of the potential drug (in this case the biological agent) must be optimized to a maximal possible intrinsic potency. In analytical development, the goal is to search for better performing reporter systems (the lowest possible κ), as analytical tool. Two different systems characterized by constants κA

20 and κB, respectively, can be compared (using the same biological agent) for their relative resistance or performance.

Complex systems involving the interaction of biological agents, such as viruses, vaccines, gene transfer vectors, antibodies proteins and living cells (either *in vitro* or *in vivo*) can be analyzed using the Hill

25 equation. A complex succession of unitary processes, each of them susceptible to be individually analyzed by the Hill equation, as a global process, can be also described by the same equation as its constitutive steps.

EXAMPLE 4

Materials and Methods

Cells:

293 human embryo kidney (HEK) cells, obtained from ATCC, were

5   cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l

glucose (DMEM; GIBGO-BRL) 10 % fetal bovine serum (FBS, Hyclone).

Hela rep-cap 32 cells, described above, were obtained from Anna Salvetti

(CHU, Nantes) and cultured in the medium described above.

Plasmids:

10   pNB-Adeno, which encodes the entire E2A and E4 regions and VA

RNA I and II genes of Adenovirus type 5, was constructed by ligating into

the polylinker of multiple cloning site of pBSII KS (+/-) (Stratagene, San

Diego, USA) the SalI-HindIII fragment (9842-11555 nt) of Adenovirus

type 5) and the BamHI-ClaI fragment (21563- 35950) of pBR325. All

15   fragments of adenovirus gene were obtained from the plasmid pBHG-10

(Microbix, Ontario, Canada). pNB-AAV encodes the genes rep and cap of

AAV-2 was constructing by ligation of XbaI-XbaI PCR fragment

containing the genome of AAV-2 from nucleotide 200 to 4480 into XbaI

site of polylinker MCS of pBSIIKS(+/-). The PCR fragment was obtained

20   from pAV1 (ATCC, USA). Plasmid pNB-AAV was derived from plasmid

pVA1I, which contains the AAV genomic region, rep and cap. pNB-AAV

does not contain the AAV ITR's present in pAV1. pAAV-CMV(nls)LacZ

was provided by Dr Anna Salvetti (CHU, Nantes).

pCMV(nls)LacZ (rAAV vector plasmid) and pNB-Adeno were

25   prepared on DH5a *E. coli* and purified by Nucleobond AX PC500 Kit

(Macherey-Nagel), according to standard procedures. Plasmid pAAV-

CMV(nls)LacZ is derived from plasmid psub201 by deleting the rep-cap

region with SnaB I and replacing it with an expression cassette harboring

the cytomegalovirus (CMV) immediate early promoter (407 bp), the

30   nuclear localized β-galactosidase gene and the bovine growth hormone

polyA signal (324 bp) (see, Chadeuf *et al.* (2000) *J. Gene Med.* 2:260-268. pAAV-CMV(nls)LacZ was provided by Dr Anna Salvetti.

Virus:

Wild type adenovirus (AV) type 5 stock, originally provided by Dr Philippe Moullier (CHU, Nantes), was produced accordingly to standard procedures.

Construction of Rep mutant libraries

25 pmol of each mutagenic primer was placed into a 96 PCR well plate. 15 μl of reaction mix (0.25 pmol of pNB-AAV), 25 pmol of the selection primer (changing one non-essential unique restriction site to a new restriction site), 2 μl of 10 X mutagenesis buffer (100 mM Tris-acetate pH7.5, 100 mM MgOAc and 500 mM KOAc pH7.5) was added into each well. The samples were incubated at 98°C for 5 minutes and then immediately incubated for 5 minutes on ice. Finally, the plate was placed at room temperature for 30 minutes.

The primer extension and ligation reactions of the new strands were completed by adding to each sample: 7 μl of nucleotide mix (2.86 mM each nucleotide and 1.43 X mutagenesis buffer) and 3μl of a fresh 1:10 enzyme dilution mix (0.025 U/μl of native T7 DNA polymerase and 1 U/μl of T4 DNA ligase were diluted in 20 mM Tris HCl pH7.5, 10 mM KCl, 10 mM β- mercaptoethanol, 1 mM DTT, 0.1 mM EDTA and 50% glycerol). Samples were incubated at 37°C for 1 hour. The T4 DNA ligase was inactivated by incubating the reactions at 72°C for 15 minutes to prevent re-ligation of the digested strands during the digestion of the parental plasmid (pNB-AAV).

Each mutagenesis reaction was digested with restriction enzyme to eliminate parental plasmids: 30 μl solution containing 3 μl of 10X enzyme digestion buffer and 10 units of restriction enzyme were added to each mutagenesis reaction and incubated at 37°C for at least 3 hours.

90 μl of the *E. coli* XLmutS competent cells (Stratagene, San Diego CA; supplemented with 1.5 μl of β-mercaptoethanol to a final concen-

tration of 25 mM) were aliquoted into prechilled deep-well plates. The plates were incubated on ice for 10 minutes and swirling gently every 2 minutes.

5 A fraction of the reactions that had been digested with restriction enzyme (1/10 of the total volume) was added to the deep well plates. The plates were swirled gently prior to incubation on ice for 30 minutes. A heat pulse was performed in a 42°C water bath for 45 seconds, the transformation mixture was incubated on ice for 2 minutes and 0.45 ml of preheated SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract,

10 8.5 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$ and 20 mM glucose at pH 7) was added. The plates were incubated at 37°C for 1 hour with shaking.

To enrich for mutant plasmids, 1 ml of 2X YT broth medium (YT medium is 0.5% yeast extract, 0.5% NaCl, 0.8% bacto-tryptone), supplemented with 100 µg/ml of ampicillin, was added to each

15 transformation mixture and the cultures were grown overnight at 37°C with shaking. Plasmid DNA isolation was performed from each mutant culture using standard procedure described in Nucleospin Multi-96 Plus Plasmid Kit (Macherey-Nagel). Five hundred µg of the resulting isolated DNA was digested with 10 units of the selection restriction enzyme in a

20 total volume of 30µl containing 3 µl of 10X enzyme digestion buffer for overnight at 37°C.

A fraction of the digested reactions (1/10 of the total volume) were transformed into 40 µl of Epicurian coli XL1-Blue competent cells supplemented with 0.68 µl of β-mercaptoethanol to a final concentration

25 of 25 mM. After heat pulse, 0.45 ml of SOC was added and the transformation mixtures were incubated for 1 hour at 37°C with shaking before to be plate on LB-ampicillin agar plates. The agar plates were incubated overnight at 37°C and the colonies obtained were picked up and grown overnight at 37°C into deep-well plates.

30 Four clones per reaction were screened for the presence of the mutation using restriction enzyme specific to the new restriction site

introduced into the mutated plasmid with the selection primer. The cDNA from selected clones was also sequenced to confirm the presence of the expected mutation.

Monitoring rAAV Production

5   rAAV from each of the above wells, were produced by triple transfection on 293 HEK cells. 3 x 10^4 cells were seeded into each well of 96 micro-well plate and cultured for 24 hours before transfection. Transfection was made on cells at about 70% confluency. 25 kDa PEI (poly-ethylene-imine, Sigma-Aldrich) was used for the triple transfection

10   step. Equimolar amounts of the three plasmids AV helper plasmid (pNB-Adeno), AAV helper plasmid (pNB-AAV or a mutant clone rep plasmid) and vector plasmid (pAAV-CMV(nls)LacZ) were mixed with 10 mM PEI by gently shaking. The mixture was the added to the medium culture on the cells. 60 hours after transfection, the culture medium was replaced with

15   100 μl of lysis buffer (50 mM Hepes, pH 7.4; 150 mM NaCl; 1 m MgCl$_2$; 1 mM CaCl$_2$; 0.01% CHAPS). After one cycle of freeze-thawing the cellular lysate was filtered through a millipore filter 96 well plate and stored at −80°C.

rAAV infection particles (ip)

20   Titers of rAAV vector particles were determined on HeLa rep/cap 32 cells using standard dRA (serial dilution replication assay) test. Cells were plated 24 hours before infection at a density of 1 x 10^4 cells in 96-well plates. Serial dilutions of the rAAV preparation were made between 1 and 1 x 10^6 μl and used for co-infection of the HeLa rep/cap 32 cells

25   together with wt-AV type 5 (MOI 25). 48 hours after infection the ip were measured by real time PCR or by the quantification of biological activity of the transgene.

Real Time PCR

Infected HeLa rep/cap 32 cells were lysed with 50 μl of solution

30   (50 mM Hepes, pH 7.4; 150 mM NaCl). After one cycle of freeze-thawing

50 μl of Proteinase K (10 mg/ml) and the lysate were incubated one hour at 55°C. The enzyme was inactivated by incubation 10 min at 96°C.

For real time PCR, 0.2 μl of lysate was taken. Final volume of the reaction was 10 μl in 384 well plate using an Applied Biosystem Prism

5 7900. The primers and fluorescence probe set corresponding to the CMV promoter were as follows: CMV 1 primer 5'-TGCCAAGTACGCCCCCTAT-3' (SEQ ID No. 733) (0.2 μM) and CMV 2 primer 5'-AGGTCATGTACTGGGCATAATGC -3' (SEQ ID No. 734) (0.2 μM) ; probe VIC-Tamra 5'-TCAATGACGGTAAATGGCCCGCCT-3' (SEQ

10 ID No. 735) (0.1 μM). dRA plots were obtained by plotting the DNA copy number (obtained by real time PCR) vs. the dilution of the rAAV preparation.

β-Galactosidase activity

After 48 hours of infection, cells were treated with trypsin, and

15 100 μl of reaction solution (GalScreen Kit, Tropix) was added and incubated for one hour at 26 °C. Luminescence was measured in NorthStar (Tropix) HTS station. dRA plots were obtained plotting the intensity of β-Galatosidase activity vs. the dilution of the rAAV preparation.

20 **Mathematical Model for results analysis:**

Results were analyzed using the Hill equation-based analysis (designated NautScan[TM]; see, Patent n° 9915884, 1999, France; published as International PCT application No. WO 01/44809 (PCT n° PCT/FR00/03503, Dec, 2000, see EXAMPLE above). Briefly, data were

25 processed using a Hill equation-based model that allows extraction of key feature indicators of performance for each individual mutant. Mutants were ranked based on the values of their individual performance and those at the top of the ranking list were selected as Leads.

Results

Generation of diversity.

To identify candidate amino acid (aa) positions on the rep protein involved in rep protein activity an Ala-scan was performed on the rep

5   sequence. For this, each amino acid in the rep protein sequence was replaced with Alanine. To do this sets of rAAV that encode mutant rep proteins in which each differs from wild type by replacement of one amino acid with Ala, was generated. Each set of rAAV was individually introduced into cells in a well of a microtiter plate, under conditions for

10 expression of the rep protein. The amount of virus that could be produced from each variant was measured as described below. Briefly, activity of Rep was assessed by determining the amount of AAV or rAAV produced using infection assays on HeLa Rep-cap 32 cells and by measurement of AAV DNA replication using Real Time PCR, or by

15 assessing transgene (β-galactosidase) expression. The relative activity of each individual mutant compared to the native protein was assessed and "hits" identified. Hit positions are the positions in the mutant proteins that resulted in an alteration (selected to be at least about 20%), in this instance all resulted in a decrease, in the amount of virus produced

20 compared to the activity of the native (wildtype) gene (see Fig. 3A).

The hits were then used for identification of leads (see, Fig. 3B). Assays for Rep activity were performed as described for identification of the hit positions. Hit positions on Rep proteins and the effect of specific amino acids on the productivity of AAV-2 summarized in the following

25 table:

Hit position	replacing amino acid (effect)	
4 (ttt) F	(gct) A (decrease)	
10 (aag) K	(gcg) A (decrease)	
20 (ccc) P	(gcc) A (decrease)	
22 (att) I	(gct) A (decrease)	
28 (tgg) W	(gcg) A (decrease)	
32 (gag) E	(gcg) A (decrease)	
38 (ccg) P	(gcg) A (decrease)	
39 (cca) P	(gca) A (decrease)	
54 (ctg) L	(gct) A (decrease)	
59 (ctg) L	(gcg) A (decrease)	
64 (ctg) L	(gcg) A (decrease)	
74 (ccg) P	(gcg) A (decrease)	
86 (gag) E	(gcg) A (decrease)	
88 (tac) Y	(gcc) A (decease)	
101 (aaa) K	(gca) A (decrease)	
124 (atc) I	(gcc) A (decrease)	
125 (gag) E	(gcg) A (decrease)	
127 (act) T	(gct) A (decrease)	
132 (ttc) F	(gcc) A (decrease)	
140 (ggc) G	(gcc) A (decrease)	
161 (acc) T	(gcc) A (decrease)	
163 (cct) P	(gct) A (decrease)	
175 (tat) Y	(gct) A (decrease)	
193 (ctg) L	(gcg) A (decrease)	
196 (gtg) V	(gcg) A (decrease)	
197 (tcg) S	(gcc) A (decrease)	
221 (tca) S	(gca) A (decrease)	
228 (gtc) V	(gcg) A (decrease)	

Hit position	replacing amino acid (effect)	
231 (ctc) L	(gcc) A (decrease)	
234 (aag) K	(gcg) A (decrease)	
237 (acc) T	(gcc) A (decrease)	
250 (tac) Y	(gcc) A (decrease)	
258 (aac) N	(gcc) A (decrease)	
260 (cgg) R	(gcg) A (decrease)	
263 (atc) I	(gcc) A (decrease)	
264 (aag) K	(gcg) A (decrease)	
334 (ggg) G	(gcg) A (decrease)	
335 (cct) V	(gct) A (decrease)	
337 (act) T	(gct) A (decrease)	
341 (acc) T	(gcc) A (decrease)	
342 (aac) N	(gcc) A (decrease)	
347 (ata) I	(gca) A (decrease)	
350 (act) T	(gct) A (decrease	(aat) N (increase)
354 (tac) Y	(gcc) A (decrease)	
363 (aac) N	(gcc) A (decrease)	
364 (ttt) F	(gct) A (decrease)	
367 (aac) N	(gcc) A (decrease)	
370 (gtc) V	(gcc) A (decrease)	
376 (tgg) W	(gcg) A (decrease)	
381 (aag) K	(gcg) A (decrease)	
382 (atg) M	(gcg) A (decrease)	
389 (tcg) S	(gcg) A (decrease)	
407 (tcc) S	(gcc) A (decrease)	
411 (ata) I	(gca) A (decrease)	
414 (act) T	(gct) A (decrease)	
420 (tcc) S	(gct) A (decrease)	

Hit position	replacing amino acid (effect)	
421 (aac) N	(gcc) A (decrease)	
422 (acc) T	(gcc) A (decrease)	
424 (atg) M	(gcg) A (decrease)	
428 (att) I	(gct) A (decrease)	
429 (gac) D	(gcc) A (decrease)	
438 (cag) Q	(gcg) A (decrease)	
440 (ccg) P	(gcg) A (decrease)	
451 (acc) T	(gcc) A (decrease)	
460 (aag) K	(gcg) A (decrease)	
462 (acc) T	(gcc) A (decrease)	(ata) I (increase)
484 (ttc) F	(gcc) A (decrease)	
488 (aag) K	(gcg) A (decrease)	
495 (ccc) P	(gcc) A (decrease)	
497 (ccc) P	(gcc) A (decrease)	(cga) R (increase)
497 (ccc) P	(gcc) A (decrease)	(ctc) L (increase)
497 (ccc) P	(gcc) A (decrease)	(tac) Y (increase)
498 (agt) S	(gct) A (decrease)	
499 (gac) D	(gcc) A (decrease)	
503 (agt) S	(gcg) A (decrease)	
511 (tca) S	(gca) A (decrease)	
512 (gtt) V	(gct) A (decrease)	
516 (tcg) S	(gcg) A (decrease)	
517 (acg) T	(gct) A (decrease)	(aac) N (increase)
518 (tca) S	(gca) A (decrease)	
519 (gac) D	(gcg) A (decrease)	
542 (ctg) L	(gcg) A (decrease)	(tcg) S (increase)
548 (aga) R	(gca) A (decrease)	(agc) S (increase)
598 (gga) G	(gca) A (decrease)	(gac) D (increase)

Hit position	replacing amino acid (effect)	
598 (gga) G	(gca) A (decrease)	(agc) S (increase)
600 (gtg) V	(gcg) A (decrease)	(ccg) P (increase)
601 (cca) P	(gca) A (decrease)	
Hit position (within intron)	replacing sequence (effect)	
630 (tgc)	gcg (decrease)	cgc or tca or cct (increase)

The hits in other AAV serotypes (see, also FIG. 4) are as follows:

HIT POSITION						
AAV-2	AAV-1	AAV-3	AAV-3B	AAV-4	AAV-6	AAV-5
4	4	4	4	4	4	4
10	10	10	10	10	10	10
20	20	20	20	20	20	20
22	22	22	22	22	22	22
29	29	29	29	29	29	29
32	32	32	32	32	32	32
38	38	38	38	38	38	38
39	39	39	39	39	39	39
54	54	54	54	54	54	54
59	59	59	59	59	59	59
64	64	64	64	64	64	64
74	74	74	74	74	74	
86	86	86	86	86	86	85
88	88	88	88	88	88	87
101	101	101	101	101	101	100
124	124	124	124	124	124	123
125	125	125	125	125	125	124
127	127	127	127	127	127	126
132	132	132	132	132	132	131
140	140	140	140	140	140	

	HIT POSITION					
AAV-2	AAV-1	AAV-3	AAV-3B	AAV-4	AAV-6	AAV-5
161	161	161	161	161	161	158
163	163	163	163	163	163	160
175	175	175	175	175	175	172
193	193	193	193	193	193	190
196	196	196	196	196	196	193
197	197	197	197	197	197	194
221	221	221	221	221	221	217
228	228	228	228	228	228	224
231	231	231	231	231	231	227
234	234	234	234	234	234	230
237	237	237	237	237	237	233
250	250	250	250	250	250	246
258	258	258	258	258	258	254
260	260	260	260	260	260	256
263	263	263	263	263	263	259
264	264	264	264	264	264	260
334	334	334	334	334	334	330
335	335	335	335	335	335	331
337	337	337	337	337	337	333
341	341	341	341	341	341	337
342	342	342	342	342	342	338
347	347	347	347	347	347	342
350	350	350	350	350	350	346
354	354	354	354	354	354	350
363	363	363	363	363	363	359
364	364	364	364	364	364	360
367	367	367	367	367	367	363
370	370	370	370	370	370	366
376	376	376	376	376	376	372
381	381	381	381	381	381	377

Row markers (left column): 5, 10, 15, 20, 25, 30

	HIT POSITION						
	AAV-2	AAV-1	AAV-3	AAV-3B	AAV-4	AAV-6	AAV-5
	382	382	382	382	382	382	378
	389	389	389	389	389	389	385
	407	407	407	407	407	407	403
	411	411	411	411	411	411	407
5	414	414	414	414	414	414	410
	420	420	420	420	420	420	416
	421	421	421	421	421	421	417
	422	422	422	422	422	422	418
	424	424	424	424	424	424	420
10	428	428	428	428	428	428	424
	429	429	429	429	429	429	425
	438	438	438	438	438	438	434
	440	440	440	440	440	440	436
	451	451	451	451	451	451	447
15	460	460	460	460	460	460	456
	462	462	462	462	462	462	458
	484	484	484	484	484	484	480
	488	488	488	488	488	488	484
	495	495	495	495	495	495	491
20	497	497	497	497	497	497	493
	498	498	498	498	498	498	494
	499	499	499	499	499	499	495
	503	503	503	503	503	503	499
	511	511	511	511	511	511	529
25	512	512	512	512	512	512	530
	516	516	516	516	516	516	534
	517	517	517	517	517	517	535
	518	518	518	518	518	518	536
	519	519	519	519	519	519	537
30	542	543	542	542	542	543	561

HIT POSITION						
AAV-2	AAV-1	AAV-3	AAV-3B	AAV-4	AAV-6	AAV-5
548	549	548	548	548	549	567
598	599	600	600	599	599	
600	602	603	603	602	602	589
601	603	604	604	603	603	590

Sets of nucleic acids encoding the rep protein were generated. The rep proteins encoded by these sets of nucleic acid molecules were those in which each amino acid position identied as a "hit" in the ala-scan step, were each sequentially replaced by all remaining 18 amino acids using site directed mutagenesis. Each mutant was designed, generated, processed and analyzed physically separated from the others in addressable arrays. No mixtures, pools, nor combinatorial processing were used.

As in the first round (alanine scan), a library of mutant rAAV was generated in which each individual mutant was independently and individually generated in a independent reaction and such that each mutant contains only a single amino acid change and this for each amino acid residue. Again, each resulting mutant rep protein was then expressed and the amount of virus produced in cells assessed and compared to the native protein.

Lead identification

Since rep proteins that result in increased virus production are of interest, those mutants that lead to an increase in the amount of virus produced (2 to 10 times the native activity), were selected as "leads." Ten such mutants were identified.

Based on the results obtained from the assays described above (i.e. titer of virus produced by each rep variant), each individual rep variant was assigned a specific activity. Those variant proteins displaying the highest titers were selected as leads (see Table above). Leads include:

amino acid replacement of T by N at Hit position 350; T by I at Hit position 462; P by R at Hit position 497; P by L at Hit position 497; P by Y at Hit position 497; T by N at Hit position 517; G by S at Hit position 598; G by D at Hit position 598; V by P at Hit position 600.

5   Also provided are combinations of the above mutant Rep 78, 68, 52. 40 proteins, nucleic acids encoding the proteins, and recombinant AAV (any serotype) contains the mutation at the indicated position or corresponding position for serotypes other than AAV-2, including any set forth in the following table and corresponding SEQ ID Nos. Each amino

10   acid sequence is set forth in a separate sequence ID listing; for each mutation or combination thereof there is a single SEQ ID setting forth the unspliced nucleic acid sequence for Rep78/68, which for all mutations from amino acid 228 on, includes the corresponding Rep 52 and Rep 40 encoding sequence as well.

15   **Amino acid sequences of exemplary mutant Rep proteins**

Seq no.	gene	position(s)	codon(s)
seq.1	rep78	4	GCT
seq.2	rep68	4	GCT
seq.3	rep78	10	GCG
seq.4	rep68	10	GCG
seq.5	rep78	20	GCC
seq.6	rep68	20	GCC
seq.7	rep78	22	GCT
seq.8	rep68	22	GCT
seq.9	rep78	29	GCG
seq.10	rep68	29	GCG
seq.11	rep78	38	GCG
seq.12	rep68	38	GCG
seq.13	rep78	39	GCA
seq.14	rep68	39	GCA
seq.15	rep78	53	GCT
seq.16	rep68	53	GCT
seq.17	rep78	59	GCG
seq.18	rep68	59	GCG
seq.19	rep78	64	GCT
seq.20	rep68	64	GCT
seq.21	rep78	74	GCG
seq.22	rep68	74	GCG
seq.23	rep78	86	GCG
seq.24	rep68	86	GCG
seq.25	rep78	88	GCC
seq.26	rep68	88	GCC
seq.27	rep78	101	GCA

seq.28	rep68	101	GCA
seq.29	rep78	124	GCC
seq.30	rep68	124	GCC
seq.31	rep78	125	GCG
5 seq.32	rep68	125	GCG
seq.33	rep78	127	GCT
seq.34	rep68	127	GCT
seq.35	rep78	132	GCC
seq.36	rep68	132	GCC
10 seq.37	rep78	140	GCC
seq.38	rep68	140	GCC
seq.39	rep78	161	GCC
seq.40	rep68	161	GCC
seq.41	rep78	163	GCT
15 seq.42	rep68	163	GCT
seq.43	rep78	175	GCT
seq.44	rep68	175	GCT
seq.45	rep78	193	GCG
seq.46	rep68	193	GCG
20 seq.47	rep78	196	GCC
seq.48	rep68	196	GCC
seq.49	rep78	197	GCC
seq.50	rep68	197	GCC
seq.51	rep78	221	GCA
25 seq.52	rep68	221	GCA
seq.53	rep78	228	GCG
seq.54	rep52	228	GCG
seq.55	rep68	228	GCG
seq.56	rep40	228	GCG
30 seq.57	rep78	231	GCC
seq.58	rep52	231	GCC
seq.59	rep68	231	GCC
seq.60	rep40	231	GCC
seq.61	rep78	234	GCG
35 seq.62	rep52	234	GCG
seq.63	rep68	234	GCG
seq.64	rep40	234	GCG
seq.65	rep78	237	GCC
seq.66	rep52	237	GCC
40 seq.67	rep68	237	GCC
seq.68	rep40	237	GCC
seq.69	rep78	250	GCC
seq.70	rep52	250	GCC
seq.71	rep68	250	GCC
45 seq.72	rep40	250	GCC
seq.73	rep78	258	GCC
seq.74	rep52	258	GCC
seq.75	rep68	258	GCC
seq.76	rep40	258	GCC
50 seq.77	rep78	260	GCG
seq.78	rep52	260	GCG
seq.79	rep68	260	GCG
seq.80	rep40	260	GCG
seq.81	rep78	263	GCC

	seq.82	rep52	263	GCC
	seq.83	rep68	263	GCC
	seq.84	rep40	263	GCC
	seq.85	rep78	264	GCG
5	seq.86	rep52	264	GCG
	seq.87	rep68	264	GCG
	seq.88	rep40	264	GCG
	seq.89	rep78	334	GCG
	seq.90	rep52	334	GCG
10	seq.91	rep68	334	GCG
	seq.92	rep40	334	GCG
	seq.93	rep78	335	GCT
	seq.94	rep52	335	GCT
	seq.95	rep68	335	GCT
15	seq.96	rep40	335	GCT
	seq.97	rep78	337	GCT
	seq.98	rep52	337	GCT
	seq.99	rep68	337	GCT
	seq.100	rep40	337	GCT
20	seq.101	rep78	341	GCC
	seq.102	rep52	341	GCC
	seq.103	rep68	341	GCC
	seq.104	rep40	341	GCC
	seq.105	rep78	342	GCC
25	seq.106	rep52	342	GCC
	seq.107	rep68	342	GCC
	seq.108	rep40	342	GCC
	seq.109	rep78	347	GCA
	seq.110	rep52	347	GCA
30	seq.111	rep68	347	GCA
	seq.112	rep40	347	GCA
	seq.113	rep78	350	AAT
	seq.114	rep52	350	AAT
	seq.115	rep68	350	AAT
35	seq.116	rep40	350	AAT
	seq.117	rep78	350	GCT
	seq.118	rep52	350	GCT
	seq.119	rep68	350	GCT
	seq.120	rep40	350	GCT
40	seq.121	rep78	354	GCC
	seq.122	rep52	354	GCC
	seq.123	rep68	354	GCC
	seq.124	rep40	354	GCC
	seq.125	rep78	363	GCC
45	seq.126	rep52	363	GCC
	seq.127	rep68	363	GCC
	seq.128	rep40	363	GCC
	seq.129	rep78	364	GCT
	seq.130	rep52	364	GCT
50	seq.131	rep68	364	GCT
	seq.132	rep40	364	GCT
	seq.133	rep78	367	GCC
	seq.134	rep52	367	GCC
	seq.135	rep68	367	GCC

	seq.136	rep40	367	GCC
	seq.137	rep78	370	GCC
	seq.138	rep52	370	GCC
	seq.139	rep68	370	GCC
5	seq.140	rep40	370	GCC
	seq.141	rep78	376	GCG
	seq.142	rep52	376	GCG
	seq.143	rep68	376	GCG
	seq.144	rep40	376	GCG
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	seq.146	rep52	381	GCG
	seq.147	rep68	381	GCG
	seq.148	rep40	381	GCG
	seq.149	rep78	382	GCG
15	seq.150	rep52	382	GCG
	seq.151	rep68	382	GCG
	seq.152	rep40	382	GCG
	seq.153	rep78	389	GCG
	seq.154	rep52	389	GCG
20	seq.155	rep68	389	GCG
	seq.156	rep40	389	GCG
	seq.157	rep78	407	GCC
	seq.158	rep52	407	GCC
	seq.159	rep68	407	GCC
25	seq.160	rep40	407	GCC
	seq.161	rep78	411	GCA
	seq.162	rep52	411	GCA
	seq.163	rep68	411	GCA
	seq.164	rep40	411	GCA
30	seq.165	rep78	414	GCT
	seq.166	rep52	414	GCT
	seq.167	rep68	414	GCT
	seq.168	rep40	414	GCT
	seq.169	rep78	420	GCT
35	seq.170	rep52	420	GCT
	seq.171	rep68	420	GCT
	seq.172	rep40	420	GCT
	seq.173	rep78	421	GCC
	seq.174	rep52	421	GCC
40	seq.175	rep68	421	GCC
	seq.176	rep40	421	GCC
	seq.177	rep78	422	GCC
	seq.178	rep52	422	GCC
	seq.179	rep68	422	GCC
45	seq.180	rep40	422	GCC
	seq.181	rep78	424	GCG
	seq.182	rep52	424	GCG
	seq.183	rep68	424	GCG
	seq.184	rep40	424	GCG
50	seq.185	rep78	428	GCT
	seq.186	rep52	428	GCT
	seq.187	rep68	428	GCT
	seq.188	rep40	428	GCT
	seq.189	rep78	429	GCC

	seq.190	rep52	429	GCC
	seq.191	rep68	429	GCC
	seq.192	rep40	429	GCC
	seq.193	rep78	438	GCG
5	seq.194	rep52	438	GCG
	seq.195	rep68	438	GCG
	seq.196	rep40	438	GCG
	seq.197	rep78	440	GCG
	seq.198	rep52	440	GCG
10	seq.199	rep68	440	GCG
	seq.200	rep40	440	GCG
	seq.201	rep78	451	GCC
	seq.202	rep52	451	GCC
	seq.203	rep68	451	GCC
15	seq.204	rep40	451	GCC
	seq.205	rep78	460	GCG
	seq.206	rep52	460	GCG
	seq.207	rep68	460	GCG
	seq.208	rep40	460	GCG
20	seq.209	rep78	462	GCC
	seq.210	rep52	462	GCC
	seq.211	rep68	462	GCC
	seq.212	rep40	462	GCC
	seq.213	rep78	462	ATA
25	seq.214	rep52	462	ATA
	seq.215	rep68	462	ATA
	seq.216	rep40	462	ATA
	seq.217	rep78	484	GCC
	seq.218	rep52	484	GCC
30	seq.219	rep68	484	GCC
	seq.220	rep40	484	GCC
	seq.221	rep78	488	GCG
	seq.222	rep52	488	GCG
	seq.223	rep68	488	GCG
35	seq.224	rep40	488	GCG
	seq.225	rep78	495	GCC
	seq.226	rep52	495	GCC
	seq.227	rep68	495	GCC
	seq.228	rep40	495	GCC
40	seq.229	rep78	497	GCC
	seq.230	rep52	497	GCC
	seq.231	rep68	497	GCC
	seq.232	rep40	497	GCC
	seq.233	rep78	497	CGA
45	seq.234	rep52	497	CGA
	seq.235	rep68	497	CGA
	seq.236	rep40	497	CGA
	seq.237	rep78	497	CTC
	seq.238	rep52	497	CTC
50	seq.239	rep68	497	CTC
	seq.240	rep40	497	CTC
	seq.241	rep78	497	TAC
	seq.242	rep52	497	TAC
	seq.243	rep68	497	TAC

	seq.244	rep40	497	TAC
	seq.245	rep78	498	GCT
	seq.246	rep52	498	GCT
	seq.247	rep68	498	GCT
5	seq.248	rep40	498	GCT
	seq.249	rep78	499	GCC
	seq.250	rep52	499	GCC
	seq.251	rep68	499	GCC
	seq.252	rep40	499	GCC
10	seq.253	rep78	503	GCG
	seq.254	rep52	503	GCG
	seq.255	rep68	503	GCG
	seq.256	rep40	503	GCG
	seq.257	rep78	510	GCA
15	seq.258	rep52	510	GCA
	seq.259	rep68	510	GCA
	seq.260	rep40	510	GCA
	seq.261	rep78	511	GCA
	seq.262	rep52	511	GCA
20	seq.263	rep68	511	GCA
	seq.264	rep40	511	GCA
	seq.265	rep78	512	GCT
	seq.266	rep52	512	GCT
	seq.267	rep68	512	GCT
25	seq.268	rep40	512	GCT
	seq.269	rep78	516	GCG
	seq.270	rep52	516	GCG
	seq.271	rep68	516	GCG
	seq.272	rep40	516	GCG
30	seq.273	rep78	517	GCT
	seq.274	rep52	517	GCT
	seq.275	rep68	517	GCT
	seq.276	rep40	517	GCT
	seq.277	rep78	517	AAC
35	seq.278	rep52	517	AAC
	seq.279	rep68	517	AAC
	seq.280	rep40	517	AAC
	seq.281	rep78	518	GCA
	seq.282	rep52	518	GCA
40	seq.283	rep68	518	GCA
	seq.284	rep40	518	GCA
	seq.285	rep78	519	GCG
	seq.286	rep52	519	GCG
	seq.287	rep68	519	GCG
45	seq.288	rep40	519	GCG
	seq.289	rep78	598	GCA
	seq.290	rep52	598	GCA
	seq.291	rep78	598	GAC
	seq.292	rep52	598	GAC
50	seq.293	rep78	598	AGC
	seq.294	rep52	598	AGC
	seq.295	rep78	600	GCG
	seq.296	rep52	600	GCG
	seq.297	rep78	600	CCG

	seq.298	rep52	600	CCG
	seq.299	rep78	601	GCA
	seq.300	rep52	601	GCA
	seq.301	rep78	335 420 495	GCT GCC GCC
5	seq.302	rep52	335 420 495	GCT GCC GCC
	seq.303	rep68	335 420 495	GCT GCC GCC
	seq.304	rep40	335 420 495	GCT GCC GCC
	seq.305	rep78	39 140	GCA GCC
	seq.306	rep68	39 140	GCA GCC
10	seq.307	rep78	279 428 451	GCC GCT GCC
	seq.308	rep52	279 428 451	GCC GCT GCC
	seq.309	rep68	279 428 451	GCC GCT GCC
	seq.310	rep40	279 428 451	GCC GCT GCC
	seq.311	rep78	125 237 600	GCG GCC GCG
15	seq.312	rep52	125 237 600	GCG GCC GCG
	seq.313	rep68	125 237 600	GCG GCC GCG
	seq.314	rep40	125 237 600	GCG GCC GCG
	seq.315	rep78	163 259	GCT GCG
	seq.316	rep52	163 259	GCT GCG
20	seq.317	rep68	163 259	GCT GCG
	seq.318	rep40	163 259	GCT GCG
	seq.319	rep78	17 127 189	GCG GCT GCG
	seq.320	rep68	17 127 189	GCG GCT GCG
	seq.321	rep78	350 428	GCT GCT
25	seq.322	rep52	350 428	GCT GCT
	seq.323	rep68	350 428	GCT GCT
	seq.324	rep40	350 428	GCT GCT
	seq.325	rep78	54 338 495	GCC GCC GCC
	seq.326	rep52	54 338 495	GCC GCC GCC
30	seq.327	rep68	54 338 495	GCC GCC GCC
	seq.328	rep40	54 338 495	GCC GCC GCC
	seq.329	rep78	350 420	GCT GCC
	seq.330	rep52	350 420	GCT GCC
	seq.331	rep68	350 420	GCT GCC
35	seq.332	rep40	350 420	GCT GCC
	seq.333	rep78	189 197 518	GCG GCG GCA
	seq.334	rep52	189 197 518	GCG GCG GCA
	seq.335	rep68	189 197 518	GCG GCG GCA
	seq.336	rep40	189 197 518	GCG GCG GCA
40	seq.337	rep78	468 516	GCC GCG
	seq.338	rep52	468 516	GCC GCG
	seq.339	rep68	468 516	GCC GCG
	seq.340	rep40	468 516	GCC GCG
	seq.341	rep78	127 221 350 54 140	GCT GCA GCT GCC GCC
45	seq.342	rep52	127 221 350 54 140	GCT GCA GCT GCC GCC
	seq.343	rep68	127 221 350 54 140	GCT GCA GCT GCC GCC
	seq.344	rep40	127 221 350 54 140	GCT GCA GCT GCC GCC
	seq.345	rep78	221 285	GCA GCG
	seq.346	rep52	221 285	GCA GCG
50	seq.347	rep68	221 285	GCA GCG
	seq.348	rep40	221 285	GCA GCG
	seq.349	rep78	23 495	GCT GCC
	seq.350	rep52	23 495	GCT GCC
	seq.351	rep68	23 495	GCT GCC

	seq.352	rep40	23 495	GCT GCC
	seq.353	rep78	20 54 420 495	GCC GCC GCC GCC
	seq.354	rep52	20 54 420 495	GCC GCC GCC GCC
	seq.355	rep68	20 54 420 495	GCC GCC GCC GCC
5	seq.356	rep40	20 54 420 495	GCC GCC GCC GCC
	seq.357	rep78	412 612	GCC GCG
	seq.358	rep52	412 612	GCC GCG
	seq.359	rep68	412 612	GCC GCG
	seq.360	rep40	412 612	GCC GCG
10	seq.361	rep78	197 412	GCG GCC
	seq.362	rep52	197 412	GCG GCC
	seq.363	rep68	197 412	GCG GCC
	seq.364	rep40	197 412	GCG GCC
	seq.365	rep78	412 495 511	GCC GCC GCA
15	seq.366	rep52	412 495 511	GCC GCC GCA
	seq.367	rep68	412 495 511	GCC GCC GCA
	seq.368	rep40	412 495 511	GCC GCC GCA
	seq.369	rep78	98 422	GCC GCC
	seq.370	rep52	98 422	GCC GCC
20	seq.371	rep68	98 422	GCC GCC
	seq.372	rep40	98 422	GCC GCC
	seq.373	rep78	17 127 189	GCG GCT GCG
	seq.374	rep68	17 127 189	GCG GCT GCG
	seq.375	rep78	20 54 495	GCC GCC GCC
25	seq.376	rep52	20 54 495	GCC GCC GCC
	seq.377	rep68	20 54 495	GCC GCC GCC
	seq.378	rep40	20 54 495	GCC GCC GCC
	seq.379	rep78	259 54	GCG GCC
	seq.380	rep52	259 54	GCG GCC
30	seq.381	rep68	259 54	GCG GCC
	seq.382	rep40	259 54	GCG GCC
	seq.383	rep78	335 399	GCT GCG
	seq.384	rep52	335 399	GCT GCG
	seq.385	rep68	335 399	GCT GCG
35	seq.386	rep40	335 399	GCT GCG
	seq.387	rep78	221 432	GCA GCA
	seq.388	rep52	221 432	GCA GCA
	seq.389	rep68	221 432	GCA GCA
	seq.390	rep40	221 432	GCA GCA
40	seq.391	rep78	259 516	GCG GCG
	seq.392	rep52	259 516	GCG GCG
	seq.393	rep68	259 516	GCG GCG
	seq.394	rep40	259 516	GCG GCG
	seq.395	rep78	495 516	GCC GCG
45	seq.396	rep52	495 516	GCC GCG
	seq.397	rep68	495 516	GCC GCG
	seq.398	rep40	495 516	GCC GCG
	seq.399	rep78	414 14	GCT GCC
	seq.400	rep52	414 14	GCT GCC
50	seq.401	rep68	414 14	GCT GCC
	seq.402	rep40	414 14	GCT GCC
	seq.403	rep78	74 402 495	GCG GCC GCC
	seq.404	rep52	74 402 495	GCG GCC GCC
	seq.405	rep68	74 402 495	GCG GCC GCC

	seq.406	rep40	74 402 495	GCG GCC GCC
	seq.407	rep78	228 462 497	GCC GCC GCC
	seq.408	rep52	228 462 497	GCC GCC GCC
	seq.409	rep68	228 462 497	GCC GCC GCC
5	seq.410	rep40	228 462 497	GCC GCC GCC
	seq.411	rep78	290 338	GCG GCC
	seq.412	rep52	290 338	GCG GCC
	seq.413	rep68	290 338	GCG GCC
	seq.414	rep40	290 338	GCG GCC
10	seq.415	rep78	140 511	GCC GCA
	seq.416	rep52	140 511	GCC GCA
	seq.417	rep68	140 511	GCC GCA
	seq.418	rep40	140 511	GCC GCA
	seq.419	rep78	86 378	GCG GCG
15	seq.420	rep52	86 378	GCG GCG
	seq.421	rep68	86 378	GCG GCG
	seq.422	rep40	86 378	GCG GCG
	seq.423	rep78	54 86	GCC GCG
	seq.424	rep68	54 86	GCC GCG
20	seq.425	rep78	54 86	GCC GCG
	seq.426	rep68	54 86	GCC GCG
	seq.427	rep78	214 495 140	GCG GCC GCC
	seq.428	rep52	214 495 140	GCG GCC GCC
	seq.429	rep68	214 495 140	GCG GCC GCC
25	seq.430	rep40	214 495 140	GCG GCC GCC
	seq.431	rep78	495 511	GCC GCA
	seq.432	rep52	495 511	GCC GCA
	seq.433	rep68	495 511	GCC GCA
	seq.434	rep40	495 511	GCC GCA
30	seq.435	rep78	495 54	GCC GCC
	seq.436	rep52	495 54	GCC GCC
	seq.437	rep68	495 54	GCC GCC
	seq.438	rep40	495 54	GCC GCC
	seq.439	rep78	197 495	GCG GCC
35	seq.440	rep52	197 495	GCG GCC
	seq.441	rep68	197 495	GCG GCC
	seq.442	rep40	197 495	GCG GCC
	seq.443	rep78	261 20	GCC GCC
	seq.444	rep52	261 20	GCC GCC
40	seq.445	rep68	261 20	GCC GCC
	seq.446	rep40	261 20	GCC GCC
	seq.447	rep78	54 20	GCC GCC
	seq.448	rep68	54 20	GCC GCC
	seq.449	rep78	197 420	GCG GCC
45	seq.450	rep52	197 420	GCG GCC
	seq.451	rep68	197 420	GCG GCC
	seq.452	rep40	197 420	GCG GCC
	seq.453	rep78	54 338 495	GCC GCC GCC
	seq.454	rep52	54 338 495	GCC GCC GCC
50	seq.455	rep68	54 338 495	GCC GCC GCC
	seq.456	rep40	54 338 495	GCC GCC GCC
	seq.457	rep78	197 427	GCG GCG
	seq.458	rep52	197 427	GCG GCG
	seq.459	rep68	197 427	GCG GCG

	seq.460	rep40	197 427	GCG GCG
	seq.461	rep78	54 228 370 387	GCC GCC GCC GCG
	seq.462	rep52	54 228 370 387	GCC GCC GCC GCG
	seq.463	rep68	54 228 370 387	GCC GCC GCC GCG
5	seq.464	rep40	54 228 370 387	GCC GCC GCC GCG
	seq.465	rep78	221 289	GCA GCC
	seq.466	rep52	221 289	GCA GCC
	seq.467	rep68	221 289	GCA GCC
	seq.468	rep40	221 289	GCA GCC
10	seq.469	rep78	54 163	GCC GCT
	seq.470	rep68	54 163	GCC GCT
	seq.471	rep78	341 407 420	GCC GCC GCC
	seq.472	rep52	341 407 420	GCC GCC GCC
	seq.473	rep68	341 407 420	GCC GCC GCC
15	seq.474	rep40	341 407 420	GCC GCC GCC
	seq.475	rep78	54 228	GCC GCC
	seq.476	rep52	54 228	GCC GCC
	seq.477	rep68	54 228	GCC GCC
	seq.478	rep40	54 228	GCC GCC
20	seq.479	rep78	96 125 511	GCA GCG GCA
	seq.480	rep52	96 125 511	GCA GCG GCA
	seq.481	rep68	96 125 511	GCA GCG GCA
	seq.482	rep40	96 125 511	GCA GCG GCA
	seq.483	rep78	54 163	GCC GCT
25	seq.484	rep68	54 163	GCC GCT
	seq.485	rep78	197 420	GCG GCC
	seq.486	rep52	197 420	GCG GCC
	seq.487	rep68	197 420	GCG GCC
	seq.488	rep40	197 420	GCG GCC
30	seq.489	rep78	334 428 499	GCG GCT GCC
	seq.490	rep52	334 428 499	GCG GCT GCC
	seq.491	rep68	334 428 499	GCG GCT GCC
	seq.492	rep40	334 428 499	GCG GCT GCC
	seq.493	rep78	197 414	GCG GCT
35	seq.494	rep52	197 414	GCG GCT
	seq.495	rep68	197 414	GCG GCT
	seq.496	rep40	197 414	GCG GCT
	seq.497	rep78	30 54 127	GCG GCC GCT
	seq.498	rep68	30 54 127	GCG GCC GCT
40	seq.499	rep78	29 260	GCG GCG
	seq.500	rep52	29 260	GCG GCG
	seq.501	rep68	29 260	GCG GCG
	seq.502	rep40	29 260	GCG GCG
	seq.503	rep78	4 484	GCT GCC
45	seq.504	rep52	4 484	GCT GCC
	seq.505	rep68	4 484	GCT GCC
	seq.506	rep40	4 484	GCT GCC
	seq.507	rep78	258 124 132	GCC GCC GCC
	seq.508	rep52	258 124 132	GCC GCC GCC
50	seq.509	rep68	258 124 132	GCC GCC GCC
	seq.510	rep40	258 124 132	GCC GCC GCC
	seq.511	rep78	231 497	GCC GCC
	seq.512	rep52	231 497	GCC GCC
	seq.513	rep68	231 497	GCC GCC

Sequence	rep	aa position	codon
seq.514	rep40	231 497	GCC GCC
seq.515	rep78	221 258	GCA GCC
seq.516	rep52	221 258	GCA GCC
seq.517	rep68	221 258	GCA GCC
seq.518	rep40	221 258	GCA GCC
seq.519	rep78	234 264 326	GCG GCG GCC
seq.520	rep52	234 264 326	GCG GCG GCC
seq.521	rep68	234 264 326	GCG GCG GCC
seq.522	rep40	234 264 326	GCG GCG GCC
seq.523	rep78	153 398	AGC GCG
seq.524	rep52	153 398	AGC GCG
seq.525	rep68	153 398	AGC GCG
seq.526	rep40	153 398	AGC GCG
seq.527	rep78	53 216	GCG GCC
seq.528	rep68	53 216	GCG GCC
seq.529	rep78	22 382	GCT GCG
seq.530	rep52	22 382	GCT GCG
seq.531	rep68	22 382	GCT GCG
seq.532	rep40	22 382	GCT GCG
seq.533	rep78	231 411	GCC GCA
seq.534	rep52	231 411	GCC GCA
seq.535	rep68	231 411	GCC GCA
seq.536	rep40	231 411	GCC GCA
seq.537	rep78	59 305	GCG GCC
seq.538	rep52	59 305	GCG GCC
seq.539	rep68	59 305	GCG GCC
seq.540	rep40	59 305	GCG GCC
seq.541	rep78	53 231	GCG GCC
seq.542	rep52	53 231	GCG GCC
seq.543	rep68	53 231	GCG GCC
seq.544	rep40	53 231	GCG GCC
seq.545	rep78	258 498	GCC GCT
seq.546	rep52	258 498	GCC GCT
seq.547	rep68	258 498	GCC GCT
seq.548	rep40	258 498	GCC GCT
seq.549	rep78	88 231	GCC GCC
seq.550	rep52	88 231	GCC GCC
seq.551	rep68	88 231	GCC GCC
seq.552	rep40	88 231	GCC GCC
seq.553	rep78	101 363	GCA GCC
seq.554	rep52	101 363	GCA GCC
seq.555	rep68	101 363	GCA GCC
seq.556	rep40	101 363	GCA GCC
seq.557	rep78	354 132	GCC GCC
seq.558	rep52	354 132	GCC GCC
seq.559	rep68	354 132	GCC GCC
seq.560	rep40	354 132	GCC GCC
seq.561	rep78	10 132	GCG GCC
seq.562	rep68	10 132	GCG GCC

DNA Sequences

Sequence	aa position	codon
seq.563	4	GCT
seq.564	10	GCG

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	seq.565	20		GCC
	seq.566	22		GCT
	seq.567	29		GCG
	seq.568	38		GCG
5	seq.569	39		GCA
	seq.570	53		GCT
	seq.571	59		GCG
	seq.572	64		GCT
	seq.573	74		GCG
10	seq.574	86		GCG
	seq.575	88		GCC
	seq.576	101		GCA
	seq.577	124		GCC
	seq.578	125		GCG
15	seq.579	127		GCT
	seq.580	132		GCC
	seq.581	140		GCC
	seq.582	161		GCC
	seq.583	163		GCT
20	seq.584	175		GCT
	seq.585	193		GCG
	seq.586	196		GCC
	seq.587	197		GCC
	seq.588	221		GCA
25	seq.589	228 (Rep78/68)	GCG
		228 (Rep52)	GCG
		228 (Rep 40)	GCG
	seq.590	231 (Rep78/68)	GCC
		231 (Rep 52)	GCC
30		231 (Rep 40)	GCC
	seq.591	234 (Rep78/68)	GCG
		234 (Rep 52)	GCG
		234 (Rep 40)	GCG
	seq.592	237 (Rep78/68)	GCC
35		237 (Rep 52)	GCC
		237 (Rep 40)	GCC
	seq.593	250 (Rep78/68)	GCC
		250	GCC
		250	GCC
40	seq.594	258 (Rep78/68)	GCC
		258	GCC
		258	GCC
	seq.595	260 (Rep78/68)	GCG
		260	GCG
45		260	GCG
	seq.596	263 (Rep78/68)	GCC
		263	GCC
		263	GCC
	seq.597	264 (Rep78/68)	GCG
50		264	GCG
		264	GCG
	seq.598	334 (Rep78/68)	GCG
		334	GCG
		334	GCG

	seq.599	335 (Rep78/68)	GCT
		335	GCT
		335	GCT
	seq.600	337 (Rep78/68)	GCT
5		337	GCT
		337	GCT
	seq.601	341 (Rep78/68)	GCC
		341	GCC
		341	GCC
10	seq.602	342 (Rep78/68)	GCC
		342	GCC
		342	GCC
	seq.603	347 (Rep78/68)	GCA
		347	GCA
15		347	GCA
	seq.604	350 (Rep78/68)	AAT
		350	AAT
		350	AAT
	seq.605	350 (Rep78/68)	GCT
20		350	GCT
		350	GCT
	seq.606	354 (Rep78/68)	GCC
		354	GCC
		354	GCC
25	seq.607	363 (Rep78/68)	GCC
		363	GCC
		363	GCC
	seq.608	364 (Rep78/68)	GCT
		364	GCT
30		364	GCT
	seq.609	367 (Rep78/68)	GCC
		367	GCC
		367	GCC
	seq.610	370 (Rep78/68)	GCC
35		370	GCC
		370	GCC
	seq.611	376 (Rep78/68)	GCG
		376	GCG
		376	GCG
40	seq.612	381 (Rep78/68)	GCG
		381	GCG
		381	GCG
	seq.613	382 (Rep78/68)	GCG
		382	GCG
45		382	GCG
	seq.614	389 (Rep78/68)	GCG
		389	GCG
		389	GCG
	seq.615	407 (Rep78/68)	GCC
50		407	GCC
		407	GCC
	seq.616	411 (Rep78/68)	GCA
		411	GCA
		411	GCA

	seq.617	414 (Rep78/68)	GCT
		414	GCT
		414	GCT
	seq.618	420 (Rep78/68)	GCT
5		420	GCT
		420	GCT
	seq.619	421 (Rep78/68)	GCC
		421	GCC
		421	GCC
10	seq.620	422 (Rep78/68)	GCC
		422	GCC
		422	GCC
	seq.621	424 (Rep78/68)	GCG
		424	GCG
15		424	GCG
	seq.622	428 (Rep78/68)	GCT
		428	GCT
		428	GCT
	seq.623	429 (Rep78/68)	GCC
20		429	GCC
		429	GCC
	seq.624	438 (Rep78/68)	GCG
		438	GCG
		438	GCG
25	seq.625	440 (Rep78/68)	GCG
		440	GCG
		440	GCG
	seq.626	451 (Rep78/68)	GCC
		451	GCC
30		451	GCC
	seq.627	460 (Rep78/68)	GCG
		460	GCG
		460	GCG
	seq.628	462 (Rep78/68)	GCC
35		462	GCC
		462	GCC
	seq.629	462 (Rep78/68)	ATA
		462	ATA
		462	ATA
40	seq.630	484 (Rep78/68)	GCC
		484	GCC
		484	GCC
	seq.631	488 (Rep78/68)	GCG
		488	GCG
45		488	GCG
	seq.632	495 (Rep78/68)	GCC
		495	GCC
		495	GCC
	seq.633	497 (Rep78/68)	GCC
50		497	GCC
		497	GCC
	seq.634	497 (Rep78/68)	CGA
		497	CGA
		497	CGA

	seq.	position	codon
	seq.635	497 (Rep78/68)	CTC
		497	CTC
		497	CTC
	seq.636	497 (Rep78/68)	TAC
5		497	TAC
		497	TAC
	seq.637	498 (Rep78/68)	GCT
		498	GCT
		498	GCT
10	seq.638	499 (Rep78/68)	GCC
		499	GCC
		499	GCC
	seq.639	503 (Rep78/68)	GCG
		503	GCG
15		503	GCG
	seq.640	510 (Rep78/68)	GCA
		510	GCA
		510	GCA
	seq.641	511 (Rep78/68)	GCA
20		511	GCA
		511	GCA
	seq.642	512 (Rep78/68)	GCT
		512	GCT
		512	GCT
25	seq.643	516 (Rep78/68)	GCG
		516	GCG
		516	GCG
	seq.644	517 (Rep78/68)	GCT
		517	GCT
30		517	GCT
	seq.645	517 (Rep78/68)	AAC
		517	AAC
		517	AAC
	seq.646	518 (Rep78/68)	GCA
35		518	GCA
		518	GCA
	seq.647	519 (Rep78/68)	GCG
		519	GCG
		519	GCG
40	seq.648	598 (Rep78/68)	GCA
	seq.649	600 (Rep78/68)	GCG
	seq.650	601 (Rep78/68)	GCA
	seq.651	335 420 495	GCT GCC GCC
		335 420 495	GCT GCC GCC
45		335 420 495	GCT GCC GCC
	seq.652	39 140	GCA GCC
	seq.653	279 428 451	GCC GCT GCC
		279 428 451	GCC GCT GCC
		279 428 451	GCC GCT GCC
50	seq.654	125 237 600	GCG GCC GCG
		125 237 600	GCG GCC GCG
		125 237 600	GCG GCC GCG
	seq.655	163 259	GCT GCG
		163 259	GCT GCG

		163 259	GCT GCG
	seq.656	17 127 189	GCG GCT GCG
	seq.657	350 428	GCT GCT
		350 428	GCT GCT
5		350 428	GCT GCT
	seq.658	54 338 495	GCC GCC GCC
		54 338 495	GCC GCC GCC
		54 338 495	GCC GCC GCC
	seq.659	350 420	GCT GCC
10		350 420	GCT GCC
		350 420	GCT GCC
	seq.660	189 197 518	GCG GCG GCA
		189 197 518	GCG GCG GCA
		189 197 518	GCG GCG GCA
15	seq.661	468 516	GCC GCG
		468 516	GCC GCG
		468 516	GCC GCG
	seq.662	127 221 350 54 140	GCT GCA GCT GCC GCC
		127 221 350 54 140	GCT GCA GCT GCC GCC
20		127 221 350 54 140	GCT GCA GCT GCC GCC
	seq.663	221 285	GCA GCG
		221 285	GCA GCG
		221 285	GCA GCG
	seq.664	23 495	GCT GCC
25		23 495	GCT GCC
		23 495	GCT GCC
	seq.665	20 54 420 495	GCC GCC GCC GCC
		20 54 420 495	GCC GCC GCC GCC
		20 54 420 495	GCC GCC GCC GCC
30	seq.666	412 612	GCC GCG
		412 612	GCC GCG
		412 612	GCC GCG
	seq.667	197 412	GCG GCC
		197 412	GCG GCC
35		197 412	GCG GCC
	seq.668	412 495 511	GCC GCC GCA
		412 495 511	GCC GCC GCA
		412 495 511	GCC GCC GCA
	seq.669	98 422	GCC GCC
40		98 422	GCC GCC
		98 422	GCC GCC
	seq.670	17 127 189	GCG GCT GCG
	seq.671	20 54 495	GCC GCC GCC
		20 54 495	GCC GCC GCC
45		20 54 495	GCC GCC GCC
	seq.672	54 163	GCC GCT
	seq.673	259 54	GCG GCC
		259 54	GCG GCC
		259 54	GCG GCC
50	seq.674	335 399	GCT GCG
		335 399	GCT GCG
		335 399	GCT GCG
	seq.675	221 432	GCA GCA
		221 432	GCA GCA

		221 432	GCA GCA
	seq.676	259 516	GCG GCG
		259 516	GCG GCG
		259 516	GCG GCG
5	seq.677	495 516	GCC GCG
		495 516	GCC GCG
		495 516	GCC GCG
	seq.678	414 14	GCT GCC
		414 14	GCT GCC
10		414 14	GCT GCC
	seq.679	74 402 495	GCG GCC GCC
		74 402 495	GCG GCC GCC
		74 402 495	GCG GCC GCC
	seq.680	228 462 497	GCC GCC GCC
15		228 462 497	GCC GCC GCC
		228 462 497	GCC GCC GCC
	seq.681	290 338	GCG GCC
		290 338	GCG GCC
		290 338	GCG GCC
20	seq.682	140 511	GCC GCA
		140 511	GCC GCA
		140 511	GCC GCA
	seq.683	86 378	GCG GCG
		86 378	GCG GCG
25		86 378	GCG GCG
	seq.684	54 86	GCC GCG
		54 86	GCC GCG
		54 86	GCC GCG
	seq.685	214 495 140	GCG GCC GCC
30		214 495 140	GCG GCC GCC
		214 495 140	GCG GCC GCC
	seq.686	495 511	GCC GCA
		495 511	GCC GCA
		495 511	GCC GCA
35	seq.687	495 54	GCC GCC
		495 54	GCC GCC
		495 54	GCC GCC
	seq.688	197 495	GCG GCC
		197 495	GCG GCC
40		197 495	GCG GCC
	seq.689	261 20	GCC GCC
		261 20	GCC GCC
		261 20	GCC GCC
	seq.690	54 20	GCC GCC
45	seq.691	197 420	GCG GCC
		197 420	GCG GCC
		197 420	GCG GCC
	seq.692	54 338 495	GCC GCC GCC
		54 338 495	GCC GCC GCC
50		54 338 495	GCC GCC GCC
	seq.693	197 427	GCG GCG
		197 427	GCG GCG
		197 427	GCG GCG
	seq.694	54 228 370 387	GCC GCC GCC GCG

		54 228 370 387	GCC GCC GCC GCG
		54 228 370 387	GCC GCC GCC GCG
	seq.695	221 289	GCA GCC
		221 289	GCA GCC
5		221 289	GCA GCC
	seq.696	54 163	GCC GCT
		54 163	GCC GCT
	seq.697	341 407 420	GCC GCC GCC
		341 407 420	GCC GCC GCC
10		341 407 420	GCC GCC GCC
	seq.698	54 228	GCC GCC
		54 228	GCC GCC
		54 228	GCC GCC
	seq.699	96 125 511	GCA GCG GCA
15		96 125 511	GCA GCG GCA
		96 125 511	GCA GCG GCA
	seq.700	197 420	GCG GCC
		197 420	GCG GCC
		197 420	GCG GCC
20	seq.701	334 428 499	GCG GCT GCC
		334 428 499	GCG GCT GCC
		334 428 499	GCG GCT GCC
	seq.702	197 414	GCG GCT
		197 414	GCG GCT
25		197 414	GCG GCT
	seq.703	30 54 127	GCG GCC GCT
	seq.704	29 260	GCG GCG
		29 260	GCG GCG
		29 260	GCG GCG
30	seq.706	4 484	GCT GCC
		4 484	GCT GCC
		4 484	GCT GCC
	seq.707	258 124 132	GCC GCC GCC
		258 124 132	GCC GCC GCC
35		258 124 132	GCC GCC GCC
	seq.708	231 497	GCC GCC
		231 497	GCC GCC
		231 497	GCC GCC
	seq.709	221 258	GCA GCC
40		221 258	GCA GCC
		221 258	GCA GCC
	seq.710	234 264 326	GCG GCG GCC
		234 264 326	GCG GCG GCC
		234 264 326	GCG GCG GCC
45	seq.711	153 398	AGC GCG
		153 398	AGC GCG
		153 398	AGC GCG
	seq.712	53 216	GCG GCC
	seq.713	22 382	GCT GCG
50		22 382	GCT GCG
		22 382	GCT GCG
	seq.714	231 411	GCC GCA
		231 411	GCC GCA
		231 411	GCC GCA

	seq.715	59 305	GCG GCC
		59 305	GCG GCC
		59 305	GCG GCC
	seq.716	53 231	GCG GCC
5		53 231	GCG GCC
		53 231	GCG GCC
	seq.717	258 498	GCC GCT
		258 498	GCC GCT
		258 498	GCC GCT
10	seq.718	88 231	GCC GCC
		88 231	GCC GCC
		88 231	GCC GCC
	seq.719	101 363	GCA GCC
		101 363	GCA GCC
15		101 363	GCA GCC
	seq.720	354 132	GCC GCC
		354 132	GCC GCC
		354 132	GCC GCC
	seq.726	598	GAC
20	seq.727	598	AGC
	seq.728	600	CCG

The above nucleic acid molecules are provided in plasmids, which are introduced into cells to produce the encoded proteins. The analysis revealed the amino acid positions that affect Rep proteins activities.

25 Changes of amino acids at any of the hit positions result in altered protein activity. Hit positions are numbered and referenced starting from amino acid 1 (nucleotide 321 in AAV-2 genome), also codon 1 of the protein Rep78 coding sequence under control of p5 promoter of AAV-2: 4, 20, 22, 29, 32, 38, 39, 54, 59, 124, 125, 127, 132, 140, 161, 163, 193,

30 196, 197, 221, 228, 231, 234, 258, 260, 263, 264, 334, 335, 337, 342, 347, 350, 354, 363, 364, 367, 370, 376, 381, 389, 407, 411, 414, 420, 421, 422, 424, 428, 438, 440, 451, 460, 462, 484, 488, 495, 497, 498, 499, 503, 511, 512, 516, 517, 518, 542, 548, 598, 600 and 601. The encoded Rep78, Rep68, Rep 52 and Rep 40 proteins

35 and rAAV encoding the mutant proteins are provided. The corresponding nucleic acid molecules, Rep proteins, rAAV and cells containing the nucleic acid molecules or rAAV in which the native proteins are from other AAV serotypes, including, but are not limited to, AAV-1, AAV-3, AAV-3B, AAV-4, AAV-5 and AAV-6.

Other hit positions identified include: 10, 64, 74, 86, 88, 101, 175, 237, 250, 334, 429 and 519.

Also provided are nucleic acid molecules, the rAAV, and the encoded proteins in which the native amino acid at each hit position is
5   replaced with another amino acid, or is deleted, or contains additional amino acids at or adjacent to or near the hit positions. In particular the following nucleic acid molecules and rAAV that encode proteins containing the following amino acid replacements or combinations thereof: T by N at Hit position 350; T by I at Hit position 462; P by R at
10   Hit position 497; P by L at Hit position 497; P by Y at Hit position 497; T by N at Hit position 517; L by S at hit position 542; R by S at hit position 548; G by D at Hit position 598; G by S at Hit position 598; V by P at Hit position 600; in order to increase Rep proteins activities in terms on AAV or rAAV productivity. The corresponding nucleic acid molecules,
15   recombinant Rep proteins from the other serotypes and the resulting rAAV are also provided (see Figs. 5 and the above Table for the corresponding position in AAV-1, AAV-3, AAV-3B, AAV-4, AAV-5 and AAV-6).

Mutant adeno-associate virus (AAV) Rep proteins and viruses
20   encoding such proteins that include mutations at one or more of residues 64, 74, 88, 175, 237, 250 and 429, where residue 1 corresponds to residue 1 of the Rep78 protein encoding by nucleotides 321-323 of the AAV-2 genome, and where the amino acids are replaced as follows: L by A at position 64; P by A at position 74; Y by A at position 88; Y by A at
25   position 175; T by A at position 237; T by A at position 250; D by A at position 429 are provided. Nucleic acid molecules encoding these viruses and the mutant proteins are also provided.

Also provided are nucleic acid molecules produced from any of the above-noted nucleic acid molecules by any directed evolution method,
30   including, but are not limited to, re-synthesis, mutagenesis, recombination and gene shuffling and any way by combining any combination of the

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molecules, *i.e.*, one, two by one, two by two, ...n by n, where n is the number of molecules to be combined (*i.e.*, combining all together). The resulting recombinant AAV and encoded proteins are also provided.

Also provided are nucleic acid molecule in which additional amino
5   acids surrounding each hit, such as one, two, three . . . ten or more, amino acids are systematically replaced, such that the resulting Rep protein(s) has increased or decreased activity. Increased activity as assessed by increased recombinant virus production in suitable cells is of particular interest for production of recombinant viruses for use, for
10   example, in gene therapy.

Also provided are combinations of the above noted mutants in which several of the noted amino acids are changed and optionally additional amino acids surrounding each hit, such as one, two, three . . . ten or more, are replaced.

15   The nucleic acid molecules of SEQ ID Nos. 563-725 and the encoded proteins (SEQ ID Nos. 1-562 and 726-728) are also provided. Recombinant AAV and cells containing the encoding nucleic acids are provided, as are the AAV produced upon replication of the AAV in the cells.

20   Methods of *in vivo* or *in vitro* production of AAV or rAAV using any of the above nucleic acid molecules or cells for intracellular expression of rep proteins or the rep gene mutants are provided. *In vitro* production is effected using cell free systems, expression or replication and/ or virus assembly. *In vivo* production is effected in mammalian cells
25   that also contain any requisite *cis* acting elements required for packaging.

Also provided are nucleic acid molecules and rAAV (any serotype) in which position 630 (or the corresponding position in another serotype; see Figs. 5 and the table above). Changes at this position and the region around it lead to changes in the activity or in the quantities of the Rep or
30   Cap proteins and/or the amount of AAV or rAAV produced in cells transduced with AAV encoding such mutants. Such mutations include

tgc to gcg change (SEQ ID No. 721). Mutations at any position surrounding the codon position 630 that increase or decrease the Rep or Cap proteins quantities or activities are also provided. Methods using the rAAV (any serotype) that contain nucleic acid molecules with a mutation

5 at position 630 or within 1, 2, 3 . . . .10 or more bases thereof for the intracellular expression rep proteins or the rep gene mutants covered by claims 10 to 13, for the production of AAV or rAAV (either *in vitro, in vivo* or *ex vivo*) are provided. *In vitro* methods include cell free systems, expression or replication and/or virus assembly.

10 Also provided are rAAV (and other serotypes with corresponding changes) and nucleic acid molecules encoding an amino acid replacement by N at Hit position 350 of AAV- 1, AAV-3, AAV-3B, AAV-4 and AAV-6 or at Hit position 346 of AAV-5; by I at Hit position 462 of AAV-1, AAV-3, AAV-3B, AAV-4 and AAV-6 or at Hit position 458 of AAV-5; by

15 either R, L or Y at Hit position 497 of AAV-1, AAV-3, AAV-3B, AAV-4 and AAV-6 or at Hit position 493 of AAV-5; by N at Hit position 517 of AAV-1, AAV-3, AAV-3B, AAV-4 and AAV-6 or at Hit position 535 of AAV-5; by S at hit position 543 of AAV-1 and AAV-6 or at hit position 542 of AAV-3, AAV-3B and AAV-4 or at hit position 561 of AAV-5; by S

20 at hit position 549 of AAV-1 and AAV-6 or at hit position 548 of AAV-3, AAV-3B and AAV-4 or at hit position 567 of AAV-5; by either D or S at Hit position 599 of AAV-1, AAV-4 and AAV-6 or at Hit position 600 of AAV-3 and AAV-3B; by P at Hit position 602 of AAV-1, AAV-4 and AAV-6 or at hit position 603 of AAV-3 and AAV-3B or at hit position 589 of

25 AAV-5 in order to increase Rep proteins activities as assessed by AAV or rAAV productivity. Methods using such AAV for expression of the encoded proteins and production of AAV are also provided.

Since modifications will be apparent to those of skill in this art, it is
30 intended that this invention be limited only by the scope of the appended claims.